# EXPERIMENTAL RAT CARIES

## II. LOCATION, SEQUENCE AND EXTENT OF CARIOUS LESIONS PRODUCED IN THE NORWAY RAT WHEN RAISED ON A GENERALLY ADEQUATE, FINELY POWDERED, PURIFIED RATION

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ELEVEN FIGURES

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Experimental caries production in various animal species, which has been more and more successfully accomplished in recent years, will, no doubt, become an increasingly favored *in vivo* research tool in the elucidation of the caries problem.

When faced with the choice of species and dietary regimen, the type and the experimental usefulness of the animal and its carious lesions need careful consideration.

This paper describes a type of caries which, during the past three years, has been produced regularly and at will in common laboratory rats *(Mus norvegicus)* subsisting, from prenatal life to maturity, on a purified, finely powdered ration, adequate in all nutrients currently recognized as essential to growth, reproduction and general health (Sognnaes, '48a, '48b).

## EXPERIMENTAL

### *Distribution of the carious lesions*

Under the regimen referred to above, caries has been found to be extremely rare and late in occurrence in the maxillary rat molars, with a ratio of only about one lesion for every 30 found in the mandibular molars of a given litter. The fol-



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lowing description will, therefore, be illustrated by the carious lesions occurring in the lower jaw; and, because of the remarkable bilateral symmetry of caries exemplified by figures 1 to 4, it is, for the sake of clarity and brevity, desirable to limit the description to one side of the jaw only; namely, the lower right molars.

The mandibular molars of the rat have, as is indicated by the letters A to F in figures 5 and 6, a total of 6 main fissures: three in the first molar (A, B, C), two in the second (D, E), and one (F) in the third. Out of a total of 15 occlusal cusps, the first molar has 7 (no. 1 to 7, fig. 5), the second molar 5 (no. 8 to 12), and the third molar three (no. 13 to 15). The distribution and size of the fissures and cusps in the three molars seem to justify a ratio of about 5:3:2 between the occlusal area of the first, second and third molars, respectively. This ratio, within a quadrant of the jaw, would be important if one were to desire a finer division of each occlusal surface into the relative number of caries-susceptible squares. A number of fairly complicated scoring methods have been suggested in the past and will be used for refined analysis of experimental data in the future, but an attempt will be made in the present instance to provide a simple grading of 4 well-defined stages of carious destruction, which can be recorded by surface inspection and photography.

In rats subsisting on a purified ration (Sognnaes, '48a), the carious destruction has invariably been found to commence in the occlusal fissures of the mandibular molars. Of the 6 main fissures, the central ones of the first and second molars (B and D in figs. 5 and 6) are, as a rule, the first to show caries, closely followed by the distal fissure of the first molar (C). Thus the pattern of lesions, shown in figures 7 and 8, has quite consistently been the first gross evidence of caries in rats raised on the purified ration. At this step some pigmentation may be seen to extend into the walls of the neighbouring cusps but, where there is no grossly visible loss of surrounding tooth structure (as exemplified by fig. 7), we

may refer to this extent of caries as grade I, which is limited to isolated, non-spreading fissure lesions.

In figure 8 it is obvious that the same fissure lesions have extended to the walls of the surrounding cusps. One of these cusps, no. 7, is completely destroyed; 4 are partly destroyed, two in the first molar, no. 5 and no. 6, and two in the second molar, no. 10 and no. 11. Counting the area of the fissures and cusps involved, we thus arrive at a total of 10 carious areas; that is, about 10% of the total occlusal area of the mandibular right molars. This stage of destruction, which is reached by the beginning breakdown of the tooth substance of the cuspal walls surrounding the fissure lesions, may be classified as grade II.

From this stage the caries may progress to the previously mentioned fissures of the first (A), second (E), and third molars (F), as indicated in figure 9, or this may be preceded by further breakdown of the already existing lesions, as is shown in figure 10. In either case, it is seen that the total extent of caries has increased to a point where about one-third of the total occlusal surface area has been destroyed. Thus in figure 9 all of the 6 fissures are affected, altogether involving about one-third of the total occlusal surface area, and in figure 10 two of the lesions have spread to an extent of about 30% of the total area in view. Such cases, in which from 10 to 30% of the total occlusal area is involved by caries, may be referred to as grade III.

Following this stage, further destruction has been observed to proceed primarily through that part of the cusps which separate the fissures, in which case a lesion beginning in one fissure will tend to fuse with the neighbouring one in the same tooth. Such a course of events has already been noted in its incipient stage in figure 9, where the fissure lesions beginning in fissures B and C meet at the connecting point of cusps no. 5 and no. 6, and in the second molar of figure 10 where cusps no. 10 and no. 11 are broken down to a point where the lesions in fissures D and E are being bridged by caries.

From this stage the second molar is usually the first tooth to be completely destroyed, as is seen in figure 11, followed by the central and distal parts of the first molar. At this stage the third molar as well may exhibit gross destruction, extending to the cusps surrounding the fissure. Hence, in figure 11, more than half of the total area is destroyed by caries, an extent of decay which may be arbitrarily referred to as grade IV. Destruction beyond this stage, or complete breakdown of all the mandibular molars, may be observed after prolonged experimental periods, at which stage the maxillary molars are the only ones left for any further gradation of the caries intensity. The lesions in the mandibular molars are, however, more suitable for the grading of caries in experiments of short duration (two to 5 months). As grade V one may, therefore desire to classify the total destruction of the mandibular molars, which, obviously, would mean the end of masticatory function.

## COMMENTS

The lesions shown in this series of papers have been produced in rats raised on a finely powdered, purified ration (Sognnaes, '48a) and are not complicated by the mechanical fractures which made differential diagnosis more difficult in the coarse particle type of rat caries introduced by Hoppert, Webber and Canniff in 1931.

All the common lesions have consistently been found to occur in the occlusal surface of the rat molars, in contrast to the varied distribution observed in hamsters (Arnold, '42; Keyes, '46; Sognnaes, '48a). As in hamsters, the rat molars have sufficiently wide and open fissures to permit an early detection of the fissure lesions by careful inspection under a binocular microscope, without destroying the specimen by successive grindings as is necessary in the examination of the deep, narrow fissures of cotton rat molars (Shaw et al., '44). Unaltered by the process of caries examination, the rat jaws may, furthermore, be permanently preserved or im-

mediately prepared for more refined study of their histological, chemical, or physical properties.

It has been determined through histological observations, some of which were presented in the first paper of this series (Sognnaes, '48b), that there is a relationship between the extent of the lesions in width and depth. Since significant differences in the extent in width can be judged by a binocular surface inspection, or photography, without destruction of the specimens, it is felt that this means of judging the degree of carious destruction in the Norway rat may be repeated with ease and uniformity by different investigators, whether highly accustomed to dental examinations or not.

The type of caries produced in Norway rats raised on a purified ration has, in addition, proved to be more uniform in character than the lesions produced on other rations and in other rodents. In the first place, the use of a purified ration seems to result in a more even litter-mate distribution of carious lesions than the coarse particle diets used in the past (Sognnaes, '41 and others). Secondly, it has been found that male and female rats do not exhibit the sex differences in caries susceptibility which are observed in Syrian hamsters (Keyes, '46; Sognnaes, '48a). Finally, and in contrast to both hamsters and cotton rats (Shaw et al., '44), Norway rats reproduce well when subsisting on a purified ration (Sognnaes, '48a, '48b), an advantage which is of particular importance for further studies on prenatal nutrition in relation to the caries susceptibility of the offspring.

ACKNOWLEDGMENT

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## PLATE 1

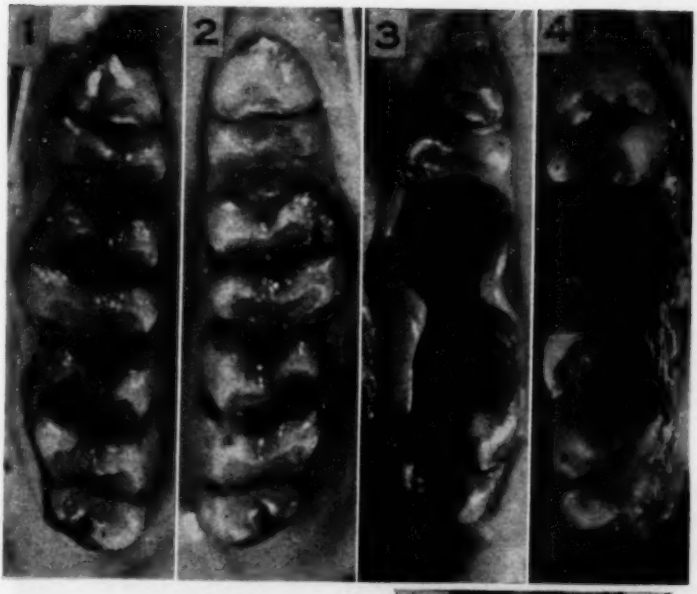
### EXPLANATION OF FIGURES

1–11 Mandibular molars of the Norway rat (raised on a finely powdered, purified ration). × 10.

1 and 2 Symmetrical bilateral distribution of a moderate degree of carious lesions in the right (1) and left (2) mandibular rat molars.

3 and 4 Bilateral symmetry of extensive carious lesions.

5 and 6 The location of the occlusal fissures and cusps of the three mandibular right rat molars.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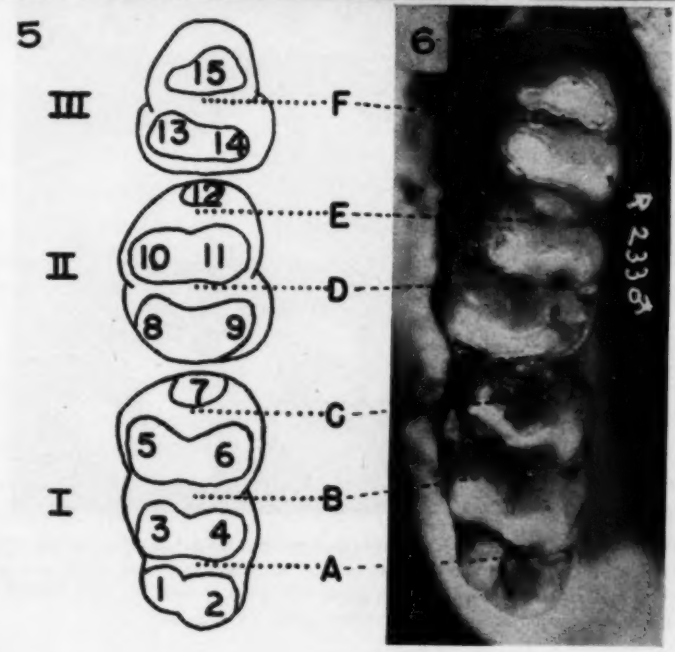
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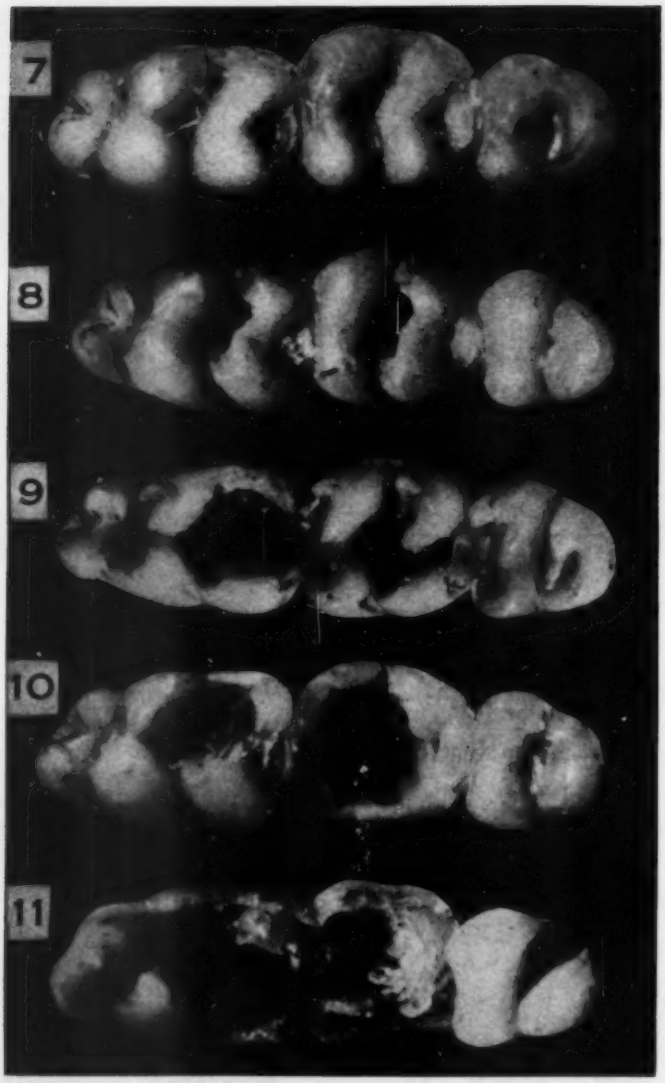


7–11 Various degrees of carious destruction. Initial fissure caries, grade 1 (fig. 7) is followed by involvment of the surrounding cuspal walls, grade II (fig. 8); of the inter-cuspal ridges, grade III (figs. 9 and 10) and, finally, by complete destruction of one or more teeth, grade IV (fig. 11).

# EXPERIMENTAL RAT CARIES

## III. THE EFFECT OF BROMIDE ON EXPERIMENTAL RAT CARIES

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FOUR FIGURES

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Previous investigations (Sognnaes, '48a, '48b, '49) have shown that experimental caries could be produced in the Norway rat when bred by mothers subsisting on a purified ration, while caries did not occur in rats bred by animals fed a stock diet composed of natural food products.

Because the purified ration used in these experiments contained all known nutritional essentials but not all food factors present in natural foods, the question arose of whether some food factor of importance to the teeth might be missing in the caries-producing diet.

When planning further experiments, it first seemed desirable to examine separately some of the volatile elements of the halogen group which could not be recovered by ashing of whole food and whose inhibitory effect upon caries has already been suggested by earlier work reviewed elsewhere (Hodge and Sognnaes, '46).

The present paper deals with the influence on rat caries of one halogen, the caries inhibitory effect of which does not seem to have been tested before; namely, the effect of bromide when added to a purified ration, during tooth development on the one hand and all through the experimental period on the other.

## MATERIALS AND METHODS

The parents of the experimental animals were selected from the same stock of Long Evans strain rats as those used in the first investigations of this series (Sognnaes, '48b, '49). From weaning time to breeding age 5 females and two males, all

TABLE 1

*Composition of the caries-producing purified ration* [1]

| BASAL MIXTURE | % |
|---|---|
| Carbohydrate (sucrose, i.e., granulated cane sugar) | 67 |
| Protein (casein) | 24 |
| Fat (corn oil) | 5 |
| Salt mixture | 4 |
| *Ash analysis of salt mixture* | |
| Calcium | 0.53% |
| Phosphorus | 0.55% |
| Magnesium | 0.033% |
| Potassium | 0.45% |
| Sodium | 0.44% |
| Chlorine | 0.76% |
| Iron | 150 p.p.m. |
| Manganese | 50 p.p.m. |
| Copper | 15 p.p.m. |
| Cobalt | 0.65 p.p.m. |
| Iodine | 20 p.p.m. |
| Zinc | 30 p.p.m. |
| Sulphur | 480 p.p.m. |

| SUPPLEMENTS | |
|---|---|
| Liver concentrate | 4% |
| *Vitamins* | |
| Thiamine | 3.5 p.p.m. |
| Riboflavin | 3.5 p.p.m. |
| Nicotinic acid | 25.0 p.p.m. |
| *p*-Aminobenzoic acid | 300.0 p.p.m. |
| Pantothenic acid | 20.0 p.p.m. |
| Pyridoxine | 3.5 p.p.m. |
| Inositol | 1,000 p.p.m. |
| Choline | 1,000 p.p.m. |
| Vitamin A | 30 U.S.P. U/gm |
| Vitamin D | 1.5 U.S.P. U/gm |

[1] The bromine groups of rats were given, in addition to the above ration, 200 μg of bromine as potassium bromide per 10 gm of diet; i.e., 20 p.p.m.

from one litter, were fed the purified ration shown in table 1 with the addition of 200 μg of bromine (as potassium bromide) per 10 gm of the purified diet, i.e., 20 parts per million. This amount, which is the same as that chosen as a high bromine supply in a nutritional study by Winnek and Smith

('37), is nearly the same as that contained in a natural non-caries-producing stock diet.[1]

After three months' subsistence on the bromide supplemented ration, the mating of the above mentioned litter mates resulted in 5 litters totalling 45 animals. These litters were at weaning time divided into two groups. One-third of these rats, i.e., a total of 15 animals, were continued on the bromide supplemented diet (group I). In the other group, an average of two-thirds of each litter, i.e., 30 animals, the addition of bromide was discontinued at that time (group II).

Three months after weaning all animals were sacrificed, except three litter pairs, which were continued on their respective diets for another two months in order to follow further progress of the existing molar lesions and to include some tests of the oral flora. (Data on the latter will be expanded and reported elsewhere.)

After sacrifice of the animals the jaws were fixed in 10% formalin, examined under binocular microscope, upon which examination the caries scoring was based, and finally prepared for histological study.

## RESULTS

The supplement of bromide during tooth development alone did not lessen the high caries susceptibility previously observed in rats bred by mothers subsisting on the purified ration, but continuation of the bromide feeding after tooth eruption did provide some inhibition and delay in the progress of caries.

In table 2 is a comparison of the two groups, one of which was continued (group I) and one discontinued (group II) on the bromide ration after eruption of the molar teeth. After three months, the animals which were continued on the bromide supplemented diet had only half as many carious areas as their litter mates from whose diet the bromide was removed at weaning. Furthermore, three out of the 12 rats in

[1] Purina laboratory chow.

TABLE 2

*The effect on rat caries of bromide added to the purified ration*

| GROUP | EXPERIMENTAL CONDITION | LITTER REFERENCE | NO. OF RATS | EXPERIMENTAL RESULTS [1] | | |
|---|---|---|---|---|---|---|
| | | | | Molars affected | Carious areas | Caries score |
| I | Continuous bromide supplement before and after eruption of molars | A | 2 | 1.5 | 1.5 | 1.5 |
| | | B | 3 | 2.0 | 2.3 | 3.0 |
| | | C | 3 | 2.6 | 2.6 | 4.0 |
| | | D | 4 [2] | 3.2 | 5.0 | 7.0 |
| | | Average for all litters | 12 | 2.5 | 3.1 | 4.3 |
| II | Bromide supplement discontinued after eruption of molars | A | 4 | 4.5 | 6.7 | 9.5 |
| | | B | 7 | 4.0 | 5.6 | 7.1 |
| | | C | 5 | 4.6 | 6.6 | 8.2 |
| | | D | 6 | 4.7 | 7.3 | 11.0 |
| | | E | 5 | 4.8 | 8.4 | 14.0 |
| | | Average for all litters | 27 | 4.5 | 6.8 | 9.9 |

[1] Based upon binocular examination (× 30) of molars following sacrifice of the rats three months after weaning.

[2] Photomicrographs in figures 3 and 4 are from the molars of one of these litter mates.

TABLE 3

*Individual litter-mate comparison of caries intensity with and without prolongation of the bromide supplement*

| GROUP | EXPERIMENTAL CONDITION | LITTER REFERENCE | EXPERIMENTAL RESULTS [1] | | |
|---|---|---|---|---|---|
| | | | Molars affected | Carious areas | Caries score |
| I | Continuous bromide supplement before and after eruption of molars | A | 3 | 3 | 3 |
| | | C | 4 | 5 | 5 [2] |
| | | E | 3 | 5 | 7 |
| | | Average | 3.3 | 4.3 | 4.6 |
| II | Bromide supplement discontinued after eruption of molars | A | 6 | 8 | 16 |
| | | C | 8 | 9 | 34 [2] |
| | | E | 8 | 21 | 46 |
| | | Average | 7.3 | 16.0 | 32.0 |

[1] The rats recorded in this table were sacrificed and examined 5 months after weaning.

[2] The molars (one quadrant of the mouth) of these animals may be compared by means of the gross photographs shown in figures 1 and 2.

group I, one from each of three litters, maintained intact teeth, while all of the 27 rats in group II developed caries. This difference becomes more marked between the litter mates which were sacrificed after 5 months. Thus, table 3 shows that hardly any further progress of the lesions occurred in those rats which were continued on the bromide diet. In the group fed the purified diet without bromide addition, the

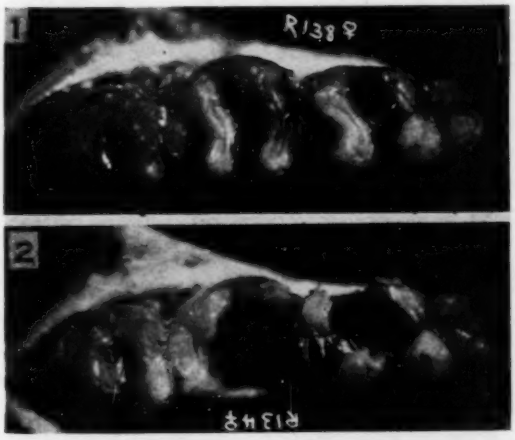


Fig. 1 Nonprogressive caries, grade I, in the mandibular left molars of female rat belonging to group I (see table 3) which was fed the purified ration with continuous bromide supplement for 5 months after weaning.

Fig. 2 Progressive caries, grade III, in the mandibular left molars of female litter mate of above, belonging to group II (see table 3), which was fed the purified ration without bromide addition for the same period after weaning.

carious score showed a three-fold increase over the previous level and was 6 times higher than that of group I. It is of interest that the severity of caries was more affected by the bromide than the incidence. The great difference between group I and II is quite consistent in each pair of litter mates, especially after 5 months, as is evident from table 3 as well as from the photographs shown in figures 1 and 2.

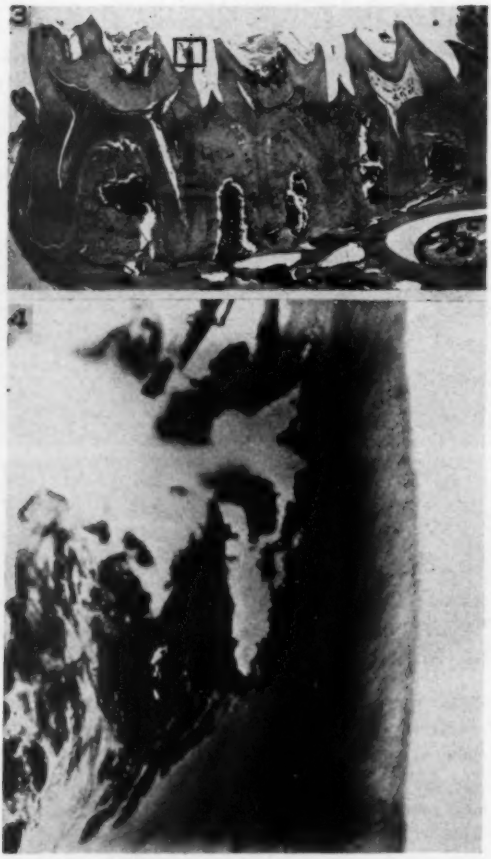

Fig. 3 Decalcified paraffin section, Masson stain (× 10), showing the mandibular molars of rat fed the purified ration with the addition of bromide for three months after weaning (see group I, table 2). The carious disintegration is walled off by a considerable layer of secondary dentine, suggesting slow progress of the lesions.

Fig. 4 Serial section from above, Gram stain (× 500), showing undermining caries of distal cusp of first molar with a dense Gram-positive layer of disintegrated dentine lining the cavity and a few areas extending into underlying dentinal tubules.

On the whole, the severity of caries was greater than expected in the animals who received the bromide addition during the period of tooth development only. Indeed, this group developed more, rather than less, caries than rats of the same strain which in the past had been fed the basic purified ration, i.e., without bromide supplement for a comparable period. It may be mentioned that two sisters of the bromide-fed mothers of groups I and II were maintained on the basic purified ration and produced litters (cousins of groups I and II) which, after a comparable experimental period, showed a caries score falling between those of groups I and II. This suggests that bromide is ineffective, if not detrimental, when administered during tooth formation alone, and that the mechanism of action of bromide in reducing caries only appears to operate when the bromide is given after tooth eruption.

## DISCUSSION

Bromine is not supposed to be a factor nutritionally essential for rats. Winnek and Smith ('37) fed rats a purified, practically bromine-free ration from weaning to 200 days of age. No significant difference in growth or reproduction was found between the bromine-depleted animals and groups fed a supplement of 20 p.p.m. of bromine as potassium bromide. In their experiments they nevertheless noted that the mothers fed the highly purified bromine-free ration did not take sufficient care of their offspring to raise the litters, as a result of which nothing is known about the effect of such a ration upon the future health of the offspring of bromine-deficient rats, i.e., the effect of a deficiency lasting over several generations. Similar criticism may perhaps be made of the negative findings available regarding the nutritional essentiality of other trace elements, such as fluorine.

Although no analysis of the bromine content of the teeth was made, it is interesting that bromine-fed rats had 4 times as high bromine content in their tissues as the animals fed a natural stock diet with similar bromine content (Winnek and Smith, '37). This suggests that bromine in a purified

diet may take the place of some other elements, possibly of other halogens, as is suggested by observations of the chlorine: bromine ratios of blood following bromide injections (Hastings et al., '32). If it is confirmed that animals which receive a bromide supplemented purified ration during tooth formation show an increase rather than a decrease in susceptibility to caries, the possibility may appropriately be considered that bromine can take the place of a factor more essential to the development of caries-resistant tooth structure.

Posteruptively, on the other hand, the presence of bromide did provide some reduction in the extent of caries. If this effect is confirmed, then bromide is the third halogen compound to show a measurable caries-inhibiting effect when added to a caries-producing diet after eruption of the teeth. In searching for a factor which would inhibit oral fermentation, Miller ('38) observed a reduction of the molar lesions in rats resulting from the addition of fluorides and iodoacetate, respectively, to a coarsely ground ration. Because the lesions produced by such a ration are probably mechanical in their initiation, it may, therefore, be assumed that Miller and others using the coarse rations (see review by Hodge and Sognnaes, '46) have observed an inhibition of the progress rather than the initiation of the lesions. Traumatic factors as contributing causes are believed to have been convincingly ruled out in the rat caries reported in this series, as is indicated in part I of this study (Sognnaes, '48b). Even so, the bromide addition to the finely powdered, purified diet also seems primarily to have reduced the progress of the lesions rather than their initiation. After three months both experimental groups were seen to have developed a fair number of lesions, but the marked difference in severity became apparent two months later. While the lesions of the bromide-fed rats were still in an incipient stage their litter mates, after discontinuation of the bromide supplement, developed very severe lesions.

At present it is not known whether the different caries-conducive effects of sucrose, dextrin, starch, and so forth, on the teeth are due to the form of carbohydrates per se or to the presence in higher carbohydrates of non-removable substances. Of such there are many besides bromine, the presence of which does not appear to be a potent enough caries inhibitor to explain the decreasing caries incidence of rats fed sucrose, dextrin, and other higher carbohydrates. It seems certain from the above findings that the presence of bromide does not explain the consistent caries freedom in rats raised on natural stock diets.[2]

In principle it is interesting, however, that a similarity has been found in the caries-inhibiting effects of compounds containing fluorine, iodine and bromine when added to caries-producing diets and fed to rats *after* eruption of the teeth. The effect of bromine appears, on the other hand, to differ from that of fluorine when given before tooth eruption; that is, during tooth formation alone. Fluorine, at least in doses large enough to cause mottling of the enamel, is believed to have some inhibitory effect upon caries even if given during tooth development only. While the mode of action requires more study in the case of fluorine, such a pre-eruptive mechanism does not seem to apply to bromine, is not known to hold for iodine, and is unlikely as regards the more widely distributed chlorine. The ratio of these elements may, however, be important if, as already suggested, the shortage of one may shift the relative retention of another.

Through experimental caries studies using a purified ration there is hope of elucidating these questions. Further work is now in progress to test the period of maximum efficiency and the permanency of the effect of fluorine on experimental rat caries produced on a purified ration. It may well be that the presumably greater caries inhibition of fluorine in contrast to bromine is due to some combination of actions operating both during the period of tooth development as well as post-eruptively in the oral environment.

[2] Such as Purina laboratory chow.

SUMMARY

1. The absence of caries previously observed in rats bred by mothers subsisting on a natural stock diet, as compared to the extensive caries produced in rats bred on a purified ration, cannot be attributed to the presence of bromine in the stock diet, because the addition of high doses of bromide to the purified ration, during tooth development, failed to provide caries protection.

2. The caries susceptibility observed in rats bred by mothers subsisting on a purified diet is not decreased but appears to be somewhat increased by the addition of bromide to the diet during the period of tooth development alone. The possibility is considered that bromine added to a purified ration may take the place of a factor more essential to the caries resistance of the developing teeth.

3. Caries could be reduced, however, when the bromide supplement was continued after eruption of the teeth. This seems to suggest that one and the same factor (at least when added to a purified diet) may be ineffective or unfavorable to the teeth during their development and still be favorable after tooth eruption.

4. It is suggested that more effective caries inhibitors may be found in food factors which exert a favorable action during tooth development as well as posteruptively.

ACKNOWLEDGMENTS

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# THE URINARY EXCRETION OF PENTOSE- AND PHOSPHORUS-CONTAINING COMPLEXES IN NUTRITIONAL MUSCULAR DYSTROPHY

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The constant urinary excretion of ribose-phosphorus-containing complexes by patients with pseudohypertrophic muscular dystrophy has recently been reported from this laboratory (Minot et al., '49). Since the report by Goettsch and Pappenheimer ('31), extensive work has repeatedly confirmed the observation that muscular weakness and deterioration histopathologically similar to progressive muscular dystrophy in man is produced when young animals are maintained on a diet deficient in vitamin E. Both conditions are characterized by excessive creatinuria, and it seemed of interest to determine whether animals with nutritional dystrophy also excrete pentose-phosphate complexes. The present paper is a report of our investigations in regard to these constituents.

## EXPERIMENTAL

Rabbits varying from 6 to 10 weeks of age at the start of the experiment were used as subjects. They were fed Goettsch and Pappenheimer's E deficient dystrophy-producing diet

[1] Some of the data contributed to this paper by this author was also incorporated in a thesis submitted by him for partial fulfillment of the requirements for a degree of Master of Science from Vanderbilt University.

number 13. To eliminate dietary changes other than the presence or absence of vitamin E, the same food mixture was fed throughout the control and experimental periods. For the control period the treatment of the diet with ether and ferric chloride to destroy vitamin E was omitted, but it was included in the preparation of all food for the experimental period.

In order to avoid the danger of obtaining urine contaminated with particles of food, no attempt was made to collect complete 24-hour specimens of urine. Single samples of urine for chemical studies were obtained by gentle pressure over the bladder or more often by catheterization. Since previous experience had demonstrated the rapid spontaneous disintegration of the complexes in which we were interested, it seemed unwise to carry out our studies of the partition of urinary phosphates on specimens of urine which had been retained in the urinary bladder for variable and unknown periods of time. Hence the plan adopted was to empty the bladder by catheterization, using the urine thus obtained for creatine and pentose studies, while phosphate partition studies were carried out immediately after a sample was obtained by a second catheterization two hours after the first.

The chemical methods employed were those which have been described in greater detail in our earlier paper (Minot et al., '49). Following preliminary treatment with yeast and Lloyd's reagent to remove glucose and other interfering substances (Van Slyke and Hawkins, '29), 10 drops of urine were tested with Benedict's qualitative sugar reagent. At first the osazone test was carried out on many samples of treated urines even though they failed to reduce Benedict's solution. Later, however, since these attempts always gave negative results, such samples as failed to reduce copper after heating for 45 min. in a boiling water bath were considered to be free of detectable amounts of pentose. Samples giving positive reduction tests were treated with phenylhydrazine for the formation of osazones. Phosphorus was determined on fresh two-hour samples of urine by the method of Lowry and Lopez ('46). Three types of determinations were run: (a)

preexisting inorganic phosphorus; (b) phosphorus released by 7-minute hydrolysis with 1 N hydrochloric acid; and (c) total urinary phosphorus following wet ashing with sulphuric and nitric acids. Creatine and creatinine determinations by the method of Folin ('14) were also carried out because creatinuria has become so well established as a criterion of the onset and progression of nutritional muscular dystrophy (Verzar, '39; Mackenzie and McCollum, '40).

Objectively the young rabbits were lively, ate well and gained weight during the first three or 4 weeks on the E-deficient diet. The first indications of deficiency were a cessation in weight gain, some loss of appetite, and a tendency to less activity. From this point on deterioration was progressive and fairly rapid. Increasing muscular weakness and wasting developed and there was a considerable loss in weight. One rabbit died prematurely at the end of 4 weeks when only early signs of muscular deterioration were apparent. At autopsy this animal was found to have an intestinal obstruction. Four others, after eating the deficient diet for periods varying from 6 to 9 weeks, became helpless and either died or were sacrificed when in a moribund condition. At autopsy, in addition to the extensive typical changes in skeletal muscles, two rabbits that died spontaneously showed acutely dilated hearts, a finding we have seen frequently in E-deficient animals and one which has been described by Houchin and Smith ('44). In one instance (rabbit 2), when signs of muscular weakness had first become unmistakable, vitamin E was restored to the diet and supplemented with parenteral injections of $\alpha$-tocopherol phosphate. All signs of muscular weakness soon disappeared and the rabbit is still living and well several months after the experiment.

Typical chemical data obtained during the control and experimental periods are presented in table 1. Many more confirmatory repetitions of the same studies were, however, carried out at approximately three-day intervals throughout the experiments. In no instance was any pentose detected either by reduction test or by the formation of osazone dur-

TABLE 1

| RABBIT NO. | TOTAL CREATININE AS CREATINE | PENTOSE | | TOTAL PHOSPHORUS IN 2-HR. SAMPLE | DISTRIBUTION OF URINARY PHOSPHORUS | | | CONDITION OF EXPERIMENTAL SUBJECT |
|---|---|---|---|---|---|---|---|---|
| | | Reduction test | Osazone | | Pre-existing inorganic P | Total organic P | "7 minute" P | |
| | % | | | mg | % | % | % | |
| 1. | 6.2 | — | — | 7.0 | 96.3 | 3.7 | 0 | Control |
| | 18.6 | + | + | 5.3 | 84.1 | 15.9 | 11.5 | 4 weeks on deficient diet, losing weight, died prematurely of intestinal obstruction |
| 2. | 8.2 | — | — | 5.8 | 95.7 | 4.3 | 0 | Control |
| | 12.5 | — | — | 15.5 | 89.7 | 10.3 | 0 | 3 weeks on deficient diet, gaining weight |
| | 33.0 | + | + | 5.4 | 88.1 | 11.9 | 8.1 | 9 weeks on deficient diet, definite weakness |
| | 14.9 | — | — | 3.3 | 92.7 | 7.2 | 0.1 | 1 month after restoration of vitamin E |
| 3. | 5.5 | — | — | 7.3 | 97.2 | 2.8 | 0 | Control |
| | 21.8 | — | — | 2.3 | 92.9 | 7.1 | 0.4 | 3 weeks on deficient diet, no symptoms |
| | 56.5 | + | + | 0.6 | 67.2 | 32.8 | 26.9 | 5 weeks on deficient diet, extreme weakness, eating nothing, died 2 days later |
| 4. | 10.0 | — | — | 2.6 | 95.9 | 4.1 | 0 | Control |
| | 7.7 | — | — | 13.9 | 94.2 | 5.8 | 0 | 3 weeks on deficient diet, gaining weight |
| | 39.6 | + | + | 11.1 | 93.7 | 6.3 | 5.4 | 5 weeks on deficient diet, has ceased to gain |
| | 45.4 | + | + | 3.2 | 91.6 | 8.4 | 3.4 | 7 weeks on deficient diet, marked weakness, not eating |
| 5. | 10.3 | 0 | 0 | 11.9 | 95.8 | 4.2 | 0 | Control |
| | 20.3 | + | + | 5.2 | 92.7 | 7.3 | 1.0 | 4 weeks on deficient diet, appears well |
| | ... | + | + | 7.5 | 93.0 | 7.0 | 4.8 | 6 weeks on deficient diet, slight weakness, loss of appetite |
| | 58.5 | + | + | 2.4 | 91.2 | 8.8 | 5.7 | 9 weeks on deficient diet, extreme weakness and emaciation, died 4 days later |
| 6. | 2.1 | 0 | 0 | 6.6 | 94.7 | 5.3 | 0 | Control |
| | 7.8 | 0 | 0 | 12.4 | 90.3 | 9.7 | 0 | 3 weeks on deficient diet, gaining weight |
| | 10.2 | 0 | 0 | 8.4 | 93.6 | 6.4 | 0 | 5 weeks on deficient diet, appears normal |
| | 34.5 | + | + | 11.0 | 95.5 | 4.5 | 3.6 | 8 weeks on deficient diet, some weakness, losing weight |
| | 44.0 | + | + | 6.5 | 91.3 | 8.7 | 3.1 | 9 weeks on deficient diet, very weak, eats nothing |

ing the control periods or the first week or two that the rabbits ate the E-deficient diet. Studies of the partition of urinary phosphate during this same interval served to detect no readily hydrolyzable (so-called 7-minute) phosphorus, although three to 5% of the total phosphorus was present in organic combination. Approximately coincident with the first objective signs of deficiency both pentose and small amounts of readily hydrolyzable phosphate appeared in the urine, and these positive findings persisted until the end of the experiment in the 5 untreated rabbits. A considerable increase in creatinuria usually preceded by several days the presence of detectable amounts of pentose-phosphorus-complexes in the urine. In the later stages of deficiency the excessive creatinuria so characteristic of muscular dystrophy was apparent in every instance. The typical changes in creatine, pentose, and phosphorus excretion which were observed with the onset of symptoms in rabbit 2 returned to normal after the restoration of vitamin E.

For purposes of further identification, several samples of crude osazones prepared from the urine of these rabbits were pooled and repeatedly recrystallized. The purified osazone melted at 156.0°C. and no reduction of melting point was noted when the osazone from rabbit urine was mixed with pure ribosazone or with that obtained from the urine of patients with pseudohypertrophic muscular dystrophy. This and other incidental evidence in regard to the precipitability of pentose complexes with mercury in freshly obtained urine, as described in our earlier paper, all indicated that the pentose-phosphorus-complexes excreted in the urine in both nutritional and clinical muscular dystrophy are probably the same complex.

## COMMENT

At the present time it is equally impossible to evaluate in nutritional and in clinical muscular dystrophy the significance of the urinary excretion of what appear to be pentnucleotides. One possibility is that the positive urinary findings are merely

evidence of a release of cellular constituents from injured cells into the blood stream and thence into the urine. If so, it must probably be granted that the injury need not be of a specific type, for the deterioration in experimental dystrophy apparently results from a lack of vitamin E, whereas no such deficiency is demonstrable in clinical muscular dystrophy (Minot and Frank, '44). On the other hand, if one indulges in the speculation that some inborn or acquired error in metabolism may be responsible for both the muscular deterioration and the abnormal urinary constituents in clinical dystrophy, the appearance of the same abnormalities in experimental dystrophy might be taken to indicate a failure in the same metabolic step in the two conditions. On this basis, the hypothetical step involved would be one for which vitamin E is essential and which fails when the vitamin is withheld, but which may also fail in the presence of adequate amounts of vitamin E from other, as yet unrecognized, causes.

## SUMMARY AND CONCLUSIONS

Studies have been carried out on urines obtained from 6 young rabbits with muscular dystrophy produced by a dietary lack of vitamin E.

Control studies were negative. With the onset of signs of muscular dysfunction, pentose and readily hydrolyzable organically bound phosphate appeared simultaneously in the urine. The isolation and identification of pentose as an osazone and the liberation of bound phosphate by 7-minute hydrolysis with 1 N HCl was considered chemical evidence of the presence of pentose-phosphorus-containing complexes apparently identical with those reported in clinical cases of progressive muscular dystrophy.

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# THE ESSENTIAL AMINO ACID (EXCEPT TRYPTOPHAN) CONTENT OF COLOSTRUM AND MILK OF THE COW AND EWE[1]

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Although there has been considerable work done on milk proteins, a review of the literature revealed only a few reports on the essential amino acid content of whole milk protein, and limited comparative data on the amino acid composition of the milk and colostrum of different species.

The amino acid content of human and cow's milk has been compared by Plimmer and Lowndes ('37), Beach et al. ('41), Williamson ('44) and Block and Bolling ('46). The whole milk data of Plimmer and Lowndes, Beach et al., and Williamson were derived from analysis of casein and other protein fractions. Block and Bolling's work included an analysis of human colostrum. Microbiological data on the amino acid content of fresh and processed whole cow's milk have recently been reported by Hodson and Krueger ('46, '47). Cow's milk and colostrum have been analyzed for tryptophan (Sutton and Esh, '48; Esh and Sutton, '48).

Colostrum appears to provide special nutrients which enhance the chance of survival of the newborn animal. It is richer in certain vitamins than milk (Dann, '33; Semb et al., '34; Pearson and Darnell, '46; Parrish et al., '49). Not only is the protein content greater, but the protein distribution

[1] Acknowledgments are made to Mary Trant, Shirley Dieterich, Lila Corley and Patricia Sparks for technical assistance with the work.

is entirely different from that of milk (Crowther and Raistrick, '16; Parrish et al. '48). Immune proteins are known to be transferred from the mother to her offspring through colostrum. The amino acid composition of these immune proteins has been determined by Smith et al. ('46, '47). In view of these considerations, and the fact that the biological value of a protein is largely determined by its essential amino acid content, it appeared desirable to carry out a comparative study of the essential amino composition of the total protein of the colostrum and milk of the cow and sheep. Information on the amino acid composition of colostrum may find application in the nutrition of calves and lambs, especially where substitutes for milk are used.

## EXPERIMENTAL

### *Methods*

Samples of colostrum were obtained from the cows within 12 hours after parturition and from the ewes within three hours after lambing. Milk was collected after the animals had been lactating for 30 days or longer. In the case of the cows, colostrum and milk samples were obtained from the same animal. The death of a few of the lambs made it impossible to obtain paired colostrum and milk samples in all cases. When this happened, milk was obtained from another ewe of the same breed which had been nursing a lamb for 30 days. The samples were immediately analyzed for total nitrogen, and acid hydrolysates were prepared within 24 hours.

Total nitrogen was determined by semi-micro Kjeldahl procedure. Crude protein was calculated by multiplying the nitrogen value by the factor 6.38.

Twenty milliliters of colostrum or 50 ml of milk were diluted with an equal volume of 12 N HCl, and then 6 N HCl was added until a total volume of 100 ml per gram of crude protein was obtained. This mixture was refluxed for 24 hours. After the removal of the excess acid by vacuum distillation,

the hydrolysates were diluted to 100 ml, filtered and stored in a refrigerator for amino acid analysis.

Microbiological methods of amino acid assay were employed. Valine, leucine, and isoleucine were determined with *Lactobacillus arabinosus* 17–5 (Kuiken et al., '43). *Leuconostoc mesenteroides* P–60 was used to determine methionine (Lyman et al., '46), arginine, lysine and phenylalanine (Kuiken and Lyman, unpublished methods). *Streptococcus faecalis* R was used to determine threonine and histidine (Lyman et al., '47). L amino acid standards were used in the assays for leucine, isoleucine, arginine, lysine and histidine; DL standards were used in the other assays.

## RESULTS AND DISCUSSION

Data for essential amino acids (except tryptophan) found in cow's colostrum and milk are given in tables 1 and 2, respectively. The data are expressed in terms of the amino acid content of the crude protein, which provides the nutritionist with the most useful description of the relative distribution of amino acids in food products. The same data for ewe's milk are given in tables 3 and 4. Table 5, which gives the ratio of the amino acid content of milk protein to that of the protein of colostrum, was prepared to facilitate comparison of these data.

Two major differences in the relative distribution of amino acids are to be noted. Milk protein from each of these animals contains about 1.4 times the amount of methionine found in the protein of colostrum but only about 0.6 as much threonine. In the case of the other amino acids the relative proportions are more nearly equal. It will be noted that an essentially identical relationship exists between the relative proportions of these amino acids in milk and colostrum for both animals. The work of Block and Bolling ('46) suggests a similar relationship between colostrum and milk in humans. According to their data, a typical mother's milk protein contained 1.3 times as much methionine as a colostrum protein sample, and 0.9 times as much threonine. These differences

TABLE 1

*Essential amino acid (except tryptophan) content of colostrum of the cow*

(Amino acid content expressed as per cent of crude protein)

| ANIMAL NUMBER | BREED | CRUDE PROTEIN (N × 6.38) | AMINO ACIDS | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Arginine | Histidine | Isoleucine | Leucine | Lysine | Methionine | Phenyl-alanine | Threonine | Valine |
| | | % | % | % | % | % | % | % | % | % | % |
| 1 | Holstein | 13.33 | 4.16 | 2.35 | 4.87 | 9.44 | 8.06 | 1.76 | 4.60 | 7.01 | 8.29 |
| 2 | | 14.16 | 4.30 | 2.22 | 4.81 | 9.49 | 8.19 | 1.76 | 4.51 | 7.36 | 8.14 |
| 3 | | 18.37 | 4.68 | 2.50 | 5.14 | 9.74 | 8.29 | 1.97 | 4.93 | 7.41 | 8.24 |
| 4 | | 15.89 | 4.34 | 2.23 | 4.62 | 8.97 | 8.52 | 1.74 | 4.60 | 6.87 | 7.26 |
| 5 | | 15.90 | 4.15 | 2.59 | 4.85 | 9.22 | 7.98 | 2.08 | 4.58 | 6.58 | 7.68 |
| 6 | | 16.69 | 4.41 | 2.49 | 4.44 | 8.67 | 7.79 | 1.80 | 4.40 | 7.14 | 7.64 |
| 7 | | 10.62 | 3.79 | 2.50 | 4.73 | 8.77 | 7.80 | 1.93 | 4.31 | 6.22 | 7.51 |
| 8 | Jersey | 11.42 | 4.43 | 2.31 | 5.03 | 9.51 | 8.42 | 1.78 | 4.64 | 7.67 | 8.01 |
| 9 | | 12.95 | 4.02 | 2.43 | 4.68 | 8.49 | 7.56 | 1.86 | 4.22 | 6.28 | 7.30 |
| 10 | | 25.23 | 4.02 | 2.36 | 4.45 | 8.72 | 8.05 | 1.83 | 4.27 | 7.25 | 7.75 |
| 11 | | 11.37 | 4.03 | 2.33 | 4.49 | 8.56 | 7.63 | 2.16 | 4.38 | 6.84 | 7.34 |
| 12 | | 14.07 | 4.51 | 2.64 | 4.41 | 9.12 | 7.88 | 2.03 | 4.50 | 6.95 | 7.66 |
| Average ± | | | 4.24 | 2.41 | 4.71 | 9.06 | 8.01 | 1.89 | 4.49 | 6.96 | 7.73 |
| Standard error | | | ± 0.073 | ± 0.038 | ± 0.069 | ± 0.122 | ± 0.087 | ± 0.052 | ± 0.057 | ± 0.128 | ± 0.104 |

TABLE 2

*Essential amino acid (except tryptophan) content of milk of the cow*

(Amino acid content expressed as per cent of crude protein)

| ANIMAL NUMBER | BREED | CRUDE PROTEIN (N × 6.38) | AMINO ACIDS | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Arginine | Histidine | Isoleucine | Leucine | Lysine | Methionine | Phenyl-alanine | Threonine | Valine |
| | | % | % | % | % | % | % | % | % | % | % |
| 1 | Holstein | 2.85 | 3.34 | 2.53 | 5.59 | 9.16 | 8.38 | 3.01 | 4.70 | 4.01 | 7.03 |
| 2 | | 3.16 | 3.20 | 2.56 | 5.36 | 8.89 | 8.11 | 2.75 | 4.57 | 4.28 | 7.27 |
| 3 | | 3.12 | 3.36 | 2.52 | 5.67 | 8.98 | 8.43 | 2.78 | 4.54 | 4.40 | 6.90 |
| 4 | | 2.60 | 3.66 | 2.86 | 6.11 | 9.98 | 9.55 | 2.75 | 5.01 | 4.55 | 7.23 |
| 5 | | 3.05 | 3.42 | 2.69 | 5.98 | 9.34 | 8.54 | 2.42 | 4.83 | 4.44 | 6.72 |
| 6 | | 3.28 | 3.54 | 2.63 | 5.59 | 8.89 | 8.25 | 2.57 | 4.88 | 4.02 | 7.01 |
| 7 | | 3.08 | 3.44 | 2.74 | 6.04 | 8.94 | 8.62 | 2.37 | 4.76 | 4.23 | 7.04 |
| 8 | Jersey | 4.29 | 3.58 | 2.81 | 6.29 | 9.32 | 8.90 | 3.06 | 4.73 | 4.53 | 7.49 |
| 9 | | 3.53 | 3.63 | 2.78 | 5.93 | 9.19 | 7.87 | 2.41 | 4.69 | 4.41 | 6.87 |
| 10 | | 3.84 | 3.64 | 2.80 | 6.18 | 9.39 | 9.19 | 2.66 | 4.86 | 4.48 | 7.41 |
| 11 | | 4.09 | 3.56 | 2.72 | 5.76 | 9.31 | 8.62 | 2.58 | 4.61 | 4.39 | 7.29 |
| 12 | | 3.86 | 3.81 | 2.78 | 5.78 | 8.96 | 7.80 | 2.42 | 4.81 | 4.35 | 7.20 |
| Average ± | | | 3.52 | 2.70 | 5.86 | 9.20 | 8.52 | 2.65 | 4.75 | 4.34 | 7.12 |
| Standard error | | | ± 0.048 | ± 0.035 | ± 0.080 | ± 0.090 | ± 0.148 | ± 0.066 | ± 0.040 | ± 0.051 | ± 0.067 |

TABLE 3

*Essential amino acid (except tryptophan) content of colostrum of the ewe*

(Amino acid content expressed as per cent of crude protein)

| ANIMAL NUMBER | BREED | CRUDE PROTEIN (N × 6.38) | AMINO ACIDS | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Arginine | Histidine | Isoleucine | Leucine | Lysine | Methionine | Phenyl-alanine | Threonine | Valine |
| | | % | % | % | % | % | % | % | % | % | % |
| 1 | Delaine Merino | 15.82 [1] | 4.25 | 2.31 | 4.44 | 9.24 | 7.48 | 1.58 | 4.36 | 7.44 | 8.19 |
| 2 | | 20.38 | 4.06 | 2.25 | 4.56 | 9.35 | 7.85 | 1.74 | 4.30 | 7.04 | 8.12 |
| 3 | | 19.87 | 3.93 | 2.38 | 4.53 | 9.00 | 7.63 | 1.48 | 4.11 | 7.56 | 7.96 |
| 4 | | 24.93 | 4.57 | 2.51 | 4.87 | 9.74 | 8.46 | 1.62 | 4.56 | 8.14 | 9.61 |
| 5 | Dorset | 17.63 [1] | 3.80 | 2.34 | 4.38 | 9.29 | 7.71 | 1.70 | 4.21 | 6.89 | 7.85 |
| 6 | | 14.51 [1] | 3.94 | 2.59 | 5.25 | 9.82 | 8.15 | 2.26 | 4.68 | 6.09 | 7.67 |
| 7 | | 22.68 [1] | 3.78 | 2.46 | 4.90 | 10.03 | 8.16 | 1.87 | 4.64 | 6.44 | 8.14 |
| 8 | Rambouillet | 11.31 [1] | 3.84 | 2.68 | 4.85 | 9.56 | 7.70 | 2.05 | 4.48 | 6.86 | 7.95 |
| 9 | | 18.96 [1] | 3.62 | 2.77 | 5.28 | 10.09 | 8.20 | 2.28 | 4.72 | 5.98 | 7.89 |
| 10 | | 20.28 | 3.84 | 2.59 | 4.65 | 10.01 | 7.81 | 2.14 | 4.78 | 6.43 | 7.93 |
| 11 | Suffolk | 14.44 | 4.00 | 2.63 | 5.19 | 10.38 | 8.48 | 2.15 | 4.49 | 6.97 | 7.87 |
| Average ± | | | 3.97 | 2.50 | 4.81 | 9.66 | 7.97 | 1.90 | 4.48 | 6.89 | 8.11 |
| Standard error | | | ± 0.085 | ± 0.050 | ± 0.098 | ± 0.130 | ± 0.123 | ± 0.087 | ± 0.066 | ± 0.196 | ± 0.157 |

[1] Colostrum and milk samples were not obtained from the same animal.

TABLE 4

*Essential amino acid (except tryptophan) content of milk of the ewe*

(Amino acid content expressed as per cent of crude protein)

| ANIMAL NUMBER | BREED | CRUDE PROTEIN (N × 6.38) | AMINO ACIDS | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Arginine | Histidine | Isoleucine | Leucine | Lysine | Methionine | Phenyl-alanine | Threonine | Valine |
| | | % | % | % | % | % | % | % | % | % | % |
| 1 | Delaine Merino | 4.71 [1] | 3.05 | 2.73 | 5.50 | 9.80 | 9.11 | 2.48 | 4.64 | 4.34 | 7.80 |
| 2 | | 4.58 | 3.28 | 2.70 | 5.64 | 9.71 | 9.09 | 2.50 | 4.75 | 4.44 | 7.33 |
| 3 | | 5.21 | 3.15 | 2.82 | 5.70 | 9.82 | 8.52 | 2.67 | 4.67 | 4.63 | 7.33 |
| 4 | | 4.58 | 3.11 | 2.90 | 5.46 | 9.79 | 8.52 | 2.37 | 4.75 | 4.29 | 7.58 |
| 5 | Dorset | 4.98 [1] | 3.13 | 2.80 | 5.54 | 9.88 | 9.01 | 2.48 | 4.63 | 4.39 | 7.88 |
| 6 | | 4.35 [1] | 3.44 | 2.72 | 5.55 | 9.69 | 8.11 | 2.69 | 4.78 | 4.47 | 6.93 |
| 7 | | 4.98 [1] | 3.26 | 2.75 | 5.51 | 10.11 | 8.50 | 2.64 | 4.61 | 4.41 | 7.44 |
| 8 | Rambouillet | 4.90 [1] | 3.08 | 2.85 | 5.61 | 9.71 | 8.24 | 2.69 | 4.92 | 4.63 | 8.16 |
| 9 | | 4.36 [1] | 3.48 | 3.03 | 6.02 | 10.41 | 8.33 | 2.76 | 5.12 | 4.43 | 7.44 |
| 10 | | 4.58 | 3.42 | 2.68 | 5.39 | 9.48 | 8.50 | 2.51 | 4.62 | 4.32 | 7.43 |
| 11 | Suffolk | 4.63 | 3.48 | 2.81 | 5.68 | 9.46 | 8.74 | 2.59 | 4.79 | 4.25 | 7.67 |
| Average ± | | | 3.26 | 2.80 | 5.60 | 9.80 | 8.61 | 2.58 | 4.75 | 4.42 | 7.54 |
| Standard error | | | ± 0.051 | ± 0.031 | ± 0.051 | ± 0.081 | ± 0.103 | ± 0.038 | ± 0.046 | ± 0.037 | ± 0.099 |

[1] Colostrum and milk samples were not obtained from the same animal.

reflect the change in protein distribution which occurs during the transition from colostrum to milk production. The immune proteins characteristic of bovine colostrum may represent as much as 50 to 60% of the total protein in colostrum (Smith, '48). Such proteins as a class representing a number of species have a very high threonine content, ranging from 7.4 to 11.1% (Smith and Greene, '47). On the other hand, they contain from 0.7 to 1.2% methionine as compared with about 2.5% in casein (Lyman et al., '46; Hodson and Krueger, '46).

TABLE 5

*Ratios which characterize the relative amino acid composition of milk and colostrum proteins*

| AMINO ACID | RATIO OF AMINO ACID CONTENT OF MILK PROTEIN TO THAT OF COLOSTRUM PROTEIN | | RATIO OF AMINO ACID CONTENT OF COW'S MILK PROTEIN TO THAT OF EWE'S MILK PROTEIN |
|---|---|---|---|
| | Cow | Ewe | |
| Arginine | 0.83 | 0.82 | 1.08 |
| Histidine | 1.12 | 1.12 | 0.96 |
| Isoleucine | 1.24 | 1.16 | 1.05 |
| Leucine | 1.02 | 1.02 | 0.94 |
| Lysine | 1.06 | 1.08 | 0.99 |
| Methionine | 1.40 | 1.36 | 1.03 |
| Phenylalanine | 1.06 | 1.08 | 1.00 |
| Threonine | 0.62 | 0.64 | 0.98 |
| Valine | 0.92 | 0.93 | 0.94 |

The ratios of the amino acid content of cow's milk protein to ewe's milk protein are also presented in table 5. It is evident from a nutritional point of view that the amino acid patterns of these two groups of mixed proteins are essentially identical. From a consideration of amino acid data alone, it would appear that these proteins are of equal nutritional value.

Relatively minor individual variations were observed in the amino acid patterns of milk and colostrum from different animals. The extreme values in almost every case fell well within 10% of the mean. Although the data are grouped according to breed, the authors do not interpret these individual

variations in terms of breed. They probably reflect minor variations among individual animals in the relative proportions of particular proteins.

The authors' data relating to the essential amino acids in cow's milk are in general agreement with values previously reported.

## SUMMARY

Colostrum and milk of the cow and ewe were assayed for arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine by microbiological methods. The milk protein from both species contained about 1.4 times as much methionine as the protein of colostrum, but only 0.6 times as much threonine. Much smaller differences were noted in the relative proportions of the other amino acids. The amino acid patterns of the milk and colostrum of the two species are very similar.

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# THE NUTRITIVE VALUE OF CANNED FOODS [1]

## I. AMINO ACID CONTENT OF FISH AND MEAT PRODUCTS

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(Received for publication July 11, 1949)

This is the first portion of the report of an inter-laboratory amino acid assay project undertaken at the University of California at Los Angeles and the University of Wisconsin. The objects of this research were: (a) to carry out a rather extensive survey of the amino acid content of some common protein foodstuffs; (b) to establish the reliability of microbiological assay techniques; and (c) to obtain information concerning the extent to which canning may affect the amino acid content of the proteins in canned foods. The results of preliminary experiments and data on those amino acids and samples not investigated in the Wisconsin laboratory are given in this part of the report. Experimental studies duplicated in the two laboratories and those carried out only at the University of Wisconsin are presented in the second part (Neilands et al., '49).

A total of 74 individual samples [2] were assayed for the 10 essential amino acids in the two laboratories. Of this total,

[1] Paper 59. This work was supported in part by funds provided by the National Canners' Association and the Can Manufacturers' Institute, and is No. XXXIII in the series of the nutritive value of canned foods. The authors are indebted to L. E. McClure and Dolores Fickes, who determined moisture, ash and nitrogen. For paper 58, see Dunn et al. ('49b).

[2] All samples were obtained through the courtesy of the National Canners' Association, the American Can Company and the Continental Can Company. Complete details on the sources of the fish and meat products, the methods of collection and preservation, and other matters are available in the files of the National Canners' Association. Many of these data have been omitted from the present paper in order to conserve space.

41 samples were assayed in both laboratories, 20 samples were assayed only at the University of Wisconsin and 16 samples were assayed only at U.C.L.A.[3] The additional amino acids aspartic acid, glutamic acid and glycine were determined in all of the samples assayed by the present investigators. Proximate analyses were made in both laboratories.

## EXPERIMENTAL

Preliminary experiments were run on fresh sardines to determine the adequacy of preservation processes and microbiological assay methods. The heads, tails and viscera were removed within a few hours after the fish were caught. The edible portions were homogenized in a Waring Blendor and frozen in dry ice. This blended material was brought to the U.C.L.A. laboratory the same day. Aliquots of the mixture were: (a) hydrolyzed with acid or base; (b) lyophilized and stored at room temperature; and (c) stored frozen at dry ice temperature.

Samples of the hydrolyzed, lyophilized and frozen (packed in dry ice) materials were forwarded to the Wisconsin laboratory where the 10 essential amino acids were determined by the methods described in part II of this report. After 44 days of storage all of these materials were assayed for 13 amino acids at U.C.L.A. No significant differences (except for methionine) in the percentages of amino acids were observed between the hydrolyzed fresh material and that stored in the lyophilized or frozen state. Therefore, freezing, shipping and storing at dry ice temperature were adopted as a general procedure in handling samples of fresh, raw materials.[4]

[3] The apparent discrepancy in the total number of samples is due to the fact that samples SP1P, SP2P and SP3P (shrimp) are included in both of the groups analyzed separately at each laboratory rather than in the common group. The reason for this is that these particular samples were accidentally drained in the Wisconsin laboratory and only the solid portions assayed.

[4] All of the fish and meat products assayed for amino acids were furnished by the National Canners' Association from raw materials collected for this purpose.

The fresh materials were cleaned and trimmed and the edible portions were put into cans and processed according to the usual commercial cannery procedures. Twenty-four to 36 cans, numbered consecutively for later identification, were collected for each type of material (only 4 cans of whole ham were provided). When raw as well as heat processed samples were to be studied, the even numbered cans were frozen immediately after sealing by packing them in dry ice and the odd numbered cans were sent to the retorts for heat processing. A few of the samples of tuna were precooked for three to 4 hours at 100 to 103°C. in accordance with commercial preparatory methods, but not heat processed in the cans. These samples were preserved at dry ice temperature and subjected to the same treatment as the raw samples. Representative cans from more than half of the samples were made available to both laboratories and the remaining samples were supplied to one or the other of the two laboratories. All raw and precooked samples were shipped in insulated containers packed with dry ice. At U.C.L.A. these samples were stored at dry ice temperature until assayed. The heat processed samples were shipped without refrigeration and those received at U.C.L.A. were stored at room temperature in the original sealed containers until ready for use. The samples analyzed are designated throughout this and the following paper by a uniform code system. The code designation consists of: (a) two capital letters indicating the type of product; (b) a number to distinguish between different samples of the same product packed at different canneries; and (c) a final "R," "RC," or "P" indicating that the material was raw, precooked, or heat processed, respectively.

Code letters for the various products and scientific names of the fish studied are as follows: MA, Atlantic mackerel, *Scomber scombris;* MP, Pacific mackerel, *Pneumatophorus diego;* SO, Atlantic sardines in oil, *Clupea harengus;* ST, Pacific sardines in tomato sauce, *Sardinops caerulea;* FA, Atlantic fish flakes, *Gadus callarius* (cod) and *Melanogrammus aeglefinus* (haddock); SC, salmon, *Oncorhynchus tscha-*

*wytscha;* SH, spiced ham; WH, whole ham; TO, tuna in oil, *Germo, Thunnus,* and *Neothunnus* species; RB, beef; and SP, shrimp. Thus, MA1R, for example, designates a sample of raw Atlantic mackerel, and SC2P a sample of heat processed salmon.

With the single exception of the shrimp samples, all analyses were carried out on the entire can contents, which included not only the actual fish or meat but also, in the case of most of the products, various additives such as water, oil, tomato sauce, salt, spices, and sugar. For this reason the proximate composition of each sample was determined, and the amino acid values found were expressed as a percentage of the crude protein present.

The sampling and assay procedures employed in the U.C.L.A. laboratory are described in the following paragraphs.

The contents of 6 cans of material (one can of whole ham) were weighed, ground in a meat grinder and mixed well to insure representative sampling. A 300-gm aliquot of the mixture was homogenized in a Waring Blendor, using measured amounts of water when necessary to facilitate blending. Aliquots were weighed immediately for acid and alkaline hydrolysis and the remaining homogenate was packed in dry ice for storage until moisture, ash and nitrogen could be determined. Preliminary to the acid hydrolysis, 50 gm of the homogenate were allowed to digest for several days with 100 ml of 12 N HCl at room temperature. Fifty milliliters of water were added and the mixture was refluxed for 20 hours on an oil bath, cooled and filtered. The relatively small amount of residue on the filter paper was washed well with distilled water. The filtrate and washings were combined, made up to volume (500 ml) and stored in the refrigerator (at 4°C.). An appropriate aliquot was neutralized and diluted to the volume required for the assay. The assay procedures were those described previously (Dunn et al., '49a). Aspartic acid, glycine, lysine and phenylalanine were determined with *Leuconostoc mesenteroides* P–60; arginine with *Lactobacillus*

*casei;* glutamic acid, isoleucine, leucine and valine with *Lactobacillus arabinosus* 17–5; and histidine, methionine and threonine with *Lactobacillus fermenti* 36.

For alkaline hydrolysis a 10-gm aliquot was mixed with a solution of 10 gm of NaOH in sufficient water to make a total volume of 50 ml. The mixture was placed in a 250-ml flask

TABLE 1

*Percentages of amino acids in identical sardine samples assayed in two laboratories (calculated as per cent of dry weight)*

| AMINO ACID | FRESH (ACID HYDROLYSATE)[1] | | | | LYOPHILIZED[2] | | FROZEN[2] | |
|---|---|---|---|---|---|---|---|---|
| | no. 1 | | no. 2 | | | | | |
| | A[3] | B[3] | A | B | A | B | A | B |
| Arginine | 3.1 | 3.1 | 3.1 | 3.1 | 2.9 | 3.1 | 2.9 | 3.2 |
| Aspartic acid | 5.1 | | 5.0 | | 4.7 | | 4.8 | |
| Glutamic acid | 7.0 | | 7.0 | | 6.7 | | 7.1 | |
| Glycine | 2.9 | | 2.9 | | | | | |
| Histidine | 3.0 | 2.9 | 3.0 | 3.0 | 2.8 | 2.7 | 2.8 | 2.8 |
| Isoleucine | 2.7 | 2.7 | 2.6 | 2.8 | 2.5 | 2.5 | 2.6 | 2.5 |
| Leucine | 4.1 | 3.9 | 4.0 | 3.9 | 3.8 | 3.8 | 3.9 | 3.8 |
| Lysine | 4.6 | 4.3 | 4.6 | 4.2 | | 4.3 | 4.5 | 4.4 |
| Methionine | 1.7 | 1.3 | 1.6 | 1.2 | 1.5 | 1.5 | 1.6 | 1.6 |
| Phenylalanine | 2.1 | 2.1 | 2.1 | 2.2 | 2.0 | 1.9 | 2.1 | 2.0 |
| Threonine | 2.4 | 2.6 | 2.4 | 2.6 | 2.2 | 2.4 | 2.3 | 2.4 |
| Tryptophan | 0.5 | 0.5 | 0.5 | 0.5 | 0.6 | 0.4 | 0.6 | 0.4[4] |
| Valine | 3.0 | 3.0 | 3.0 | 3.1 | 2.9 | 2.7 | 2.9 | 2.8 |

[1] Prepared in the authors' laboratory.

[2] Prepared in the authors' laboratory but hydrolyzed independently in each laboratory.

[3] A — U.C.L.A.
B — University of Wisconsin.

[4] The value 0.5 was found for a hydrolysate prepared according to the procedure of Kuiken et al. ('47).

covered with a beaker, autoclaved for 10 hours at 15 lb. pressure, cooled and filtered. The relatively small amount of residue on the filter paper was washed well with cold 2 N NaOH solution. The filtrate and washings were combined, made up to 100 ml and stored in the refrigerator. Aliquots were neutralized and diluted immediately before making the assays. Tryptophan was the only amino acid determined in

the alkaline hydrolysates. *Lactobacillus arabinosus* 17–5 and essentially the same assay procedure as that described by Greene and Black ('44) were employed.

## RESULTS AND DISCUSSION

Identical or closely agreeing values were found in both laboratories for each of the 10 amino acids determined in the fresh, lyophilized and frozen samples of edible sardine

TABLE 2

*Proximate analysis of canned fish and meat products* [1]

| CODE [2] | MOISTURE | FAT [3] | ASH | PROTEIN (N × 6.25) | CODE [2] | MOISTURE | FAT [3] | ASH | PROTEIN (N × 6.25) |
|---|---|---|---|---|---|---|---|---|---|
| | % | % | % | % | | % | % | % | % |
| MA5P | 73.6 | 2.71 | 2.75 | 19.5 | SP2P | 79.4 | 0.75 | 2.29 | 16.3 |
| MA6P | 74.4 | 3.87 | 2.69 | 19.3 | SP3P [4] | | 0.86 | | 14.3 |
| MP3P | 70.0 | 7.70 | 2.88 | 21.3 | SC3P [4] | | 23.7 | | 18.6 |
| MP4P | 70.1 | 4.58 | 2.86 | 22.2 | SC5P | 60.1 | 19.2 | 1.59 | 19.1 |
| SO5P | 60.5 | 16.2 | 3.30 | 22.3 | SH2P [4] | | 31.0 | | 12.4 |
| SO6P | 49.7 | 18.6 | 2.54 | 22.4 | SH3P [4] | | 23.1 | | 15.1 |
| ST4P | 64.6 | 12.1 | 2.58 | 19.7 | TO2P | 49.4 | 20.4 | 3.03 | 24.7 |
| SP1P | 77.9 | 0.63 | 4.14 | 16.8 | TO4P | 56.4 | 17.2 | 2.85 | 24.8 |

[1] Samples analyzed at U.C.L.A. only. Values for samples analyzed both at U.C.L.A. and the University of Wisconsin are given in table 1 of the second paper (Neilands et al., '49).

[2] A complete explanation of the sample code is given in the text.

[3] The authors are in debt to the University of Wisconsin Alumni Research Foundation for these values.

[4] Sample lost owing to unintentional interruption of refrigeration. Protein was estimated from nitrogen determined in the acid hydrolysate and probably, therefore, is somewhat low due to loss of nitrogen in the humin.

material (table 1). It may be concluded from these results that the microbiological procedures employed were adequate and that the amino acids in fish protein are not altered appreciably by lyophilizing or freezing the fresh material.

The percentages of moisture, ash and protein (N × 6.25) in the samples of fish and meat products analyzed only at U.C.L.A. are given in table 2. The percentages of aspartic acid, glutamic acid and glycine in the proteins of the 41

common samples are given in table 3. The percentages of 13 amino acids determined in the proteins of the fish and meat products assayed only at U.C.L.A. are given in table 4. Although there are quantitative differences between some of

TABLE 3

*Aspartic acid, glutamic acid, and glycine in fish and meat samples* [1]

| CODE [2] | ASPARTIC ACID | GLUTAMIC ACID | GLYCINE | CODE [2] | ASPARTIC ACID | GLUTAMIC ACID | GLYCINE |
|---|---|---|---|---|---|---|---|
| | % | % | % | | % | % | % |
| MA1R | 9.2 | 13.1 | 5.7 | SC1R | 9.1 | 13.2 | 6.0 |
| MA1P | 9.3 | 13.3 | 7.0 | SC2R | 9.3 | 13.6 | 6.2 |
| MA2R | 9.2 | 13.2 | 6.2 | SC1P | 9.0 | 13.0 | 6.4 |
| MA2P | 9.0 | 12.4 | 6.7 | SC2P | 8.8 | 12.6 | 5.8 |
| MP5R | 9.0 | 12.8 | 5.6 | SH1R | 8.7 | 14.3 | 6.8 |
| MP5P | 8.3 | 13.0 | 5.3 | SH1P | 7.7 | 13.1 | 6.1 |
| MP6R | 9.0 | 12.8 | 5.8 | SH6R | 8.9 | 15.8 | 6.4 |
| MP6P | 8.5 | 12.8 | 5.4 | SH6P | 8.5 | 15.8 | 6.8 |
| SR [3] | 8.7 | 12.3 | 5.1 | WH1R | 9.1 | 14.8 | 6.2 |
| SO1R | 9.2 | 13.3 | 5.6 | WH1P | 8.6 | 14.4 | 6.0 |
| SO2R | 7.7 | 11.2 | 5.3 | WH2R | 9.5 | 15.5 | 5.9 |
| SO2P | 9.2 | 13.2 | 6.4 | WH2P | 8.7 | 14.6 | 5.9 |
| ST5R | 9.2 | 13.2 | 6.6 | TO3R | 8.3 | 13.0 | 4.6 |
| ST5P | 8.0 | 11.3 | 5.6 | TO3RC | 8.8 | 13.6 | 4.3 |
| ST6R | 8.9 | 11.5 | 5.2 | TO3P | 8.4 | 13.5 | 4.5 |
| ST6RC | 9.6 | 13.5 | 5.8 | TO5R | 8.4 | 11.4 | 6.1 |
| ST6P | 8.8 | 12.0 | 4.9 | TO5RC | 8.2 | 11.8 | 5.0 |
| FA1R | 10.2 | 15.2 | 5.2 | TO5P | 8.6 | 12.2 | 4.5 |
| FA1P | 9.8 | 14.6 | 5.0 | RB1R | 9.2 | 15.1 | 5.3 |
| FA2R | 9.0 | 13.6 | 4.6 | RB1P | 8.4 | 14.0 | 5.3 |
| FA2P | 10.1 | 15.1 | 5.7 | | | | |

[1] Values given as per cent protein (N × 6.25). Percentages for 10 other amino acids determined both at U.C.L.A. and the University of Wisconsin are given in the second part of this report (Neilands et al., '49).

[2] A complete explanation of the sample code is given in the text.

[3] A sample of raw Pacific sardines.

the samples, it is of interest that in general there was uniformity of distribution of amino acids similar to that observed previously (Dunn et al., '49a) in the organs and tissues of birds and mammals. It appears that glutamic acid and aspartic acid are major constituents of animal proteins, while

some of the essential amino acids, such as methionine and tryptophan, account for only a small proportion of the weight of any of the animal proteins.[5]

It seems of particular significance that none of the 13 amino acids determined was present in significantly lower

TABLE 4

*Amino acid content of fish and meat samples*

(Values given as per cent of protein)

| CODE[1] | AMINO ACID | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Arginine | Aspartic acid | Glutamic acid | Glycine | Histidine | Isoleucine | Leucine | Lysine | Methionine | Phenylalanine | Threonine | Tryptophan | Valine |
| MA5P | 5.2 | 8.2 | 11.7 | 7.5 | 3.4 | 4.2 | 7.4 | 7.8 | 2.8 | 3.1 | 4.6 | 1.03 | 5.0 |
| MA6P | 5.1 | 7.4 | 11.6 | 6.8 | 3.2 | 4.2 | 7.2 | 7.6 | 2.8 | 3.1 | 4.7 | 0.99 | 4.9 |
| MP3P | 5.3 | 7.9 | 12.8 | 5.9 | 5.6 | 4.9 | 7.9 | 7.8 | 2.7 | 3.8 | 4.1 | 0.94 | 5.4 |
| MP4P | 4.0 | 8.1 | 12.7 | 5.5 | 5.7 | 5.0 | 7.9 | 8.0 | 2.7 | 3.7 | 4.3 | 0.99 | 5.5 |
| SO5P | 5.2 | 8.1 | 12.5 | 5.6 | 2.0 | 4.2 | 6.8 | 8.3 | 2.7 | 3.4 | 5.1 | 0.63 | 4.8 |
| SO6P | 5.1 | 8.4 | 13.1 | 4.5 | 2.1 | 4.5 | 7.1 | 7.9 | 2.8 | 3.8 | 4.2 | 0.85 | 5.4 |
| ST4P | 5.1 | 8.8 | 13.4 | 5.6 | 4.7 | 5.1 | 7.2 | 8.7 | 3.0 | 3.8 | 4.5 | 0.97 | 5.6 |
| SP1P | 8.9 | 9.7 | 16.0 | 5.6 | 1.84 | 4.9 | 8.2 | 8.1 | 2.7 | 4.0 | 4.1 | 0.94 | 4.9 |
| SP2P | 8.3 | 9.3 | 15.5 | 6.4 | 1.72 | 5.1 | 8.1 | 8.0 | 2.5 | 3.8 | 3.9 | 0.86 | 4.9 |
| SP3P | 8.4 | 8.9 | 15.0 | 7.1 | 1.68 | 4.8 | 7.8 | 8.2 | 2.7 | 3.8 | 4.3 | 1.05 | 5.1 |
| SC3P | 5.5 | 9.0 | 13.1 | 6.6 | 2.4 | 5.2 | 7.6 | 8.5 | 3.1 | 3.7 | 4.3 | 0.80 | 5.8 |
| SC5P | 5.3 | 8.5 | 12.3 | 6.4 | 2.4 | 5.0 | 7.2 | 8.1 | 3.1 | 3.6 | 4.3 | 0.79 | 5.6 |
| SH2P | 6.1 | 8.6 | 14.6 | 6.3 | 3.0 | 4.8 | 7.8 | 8.7 | 2.5 | 3.9 | 3.2 | 0.81 | 5.3 |
| SH3P | 5.8 | 8.2 | 14.1 | 6.0 | 3.0 | 4.8 | 7.5 | 8.2 | 2.3 | 3.9 | 3.1 | 0.86 | 4.8 |
| TO2P | 5.0 | 8.7 | 12.6 | 4.5 | 4.9 | 4.7 | 7.1 | 8.2 | 2.7 | 3.6 | 4.2 | 0.89 | 5.5 |
| TO4P | 5.3 | 8.6 | 13.2 | 4.6 | 7.1 | 5.2 | 7.4 | 8.6 | 2.8 | 3.9 | 4.7 | 0.76 | 5.7 |

[1] A complete explanation of the sample code is given in the text.

amounts in the canned products than in the corresponding raw samples of fish and meat products. A more complete discussion of the data obtained in both laboratories is given in the second portion of this report (Neilands et al., '49).

[5] Methionine in duck egg albumin appears to be an exception, since it comprises more than 6% of the weight of the protein (unpublished data obtained by Dunn, Shankman and Camien).

SUMMARY

A study of the amino acid composition of fish and meat products has been made jointly by the authors and by Professors Elvehjem and Strong and collaborators at the University of Wisconsin, with the cooperation of the National Canners' Association and the Can Manufacturers' Institute.

A total of 74 individual samples were assayed in the two laboratories for the 10 essential amino acids. Of this total, 41 samples were assayed in both laboratories and the others were divided into two groups, one assayed only at Wisconsin and the other at U.C.L.A. In addition, aspartic acid, glutamic acid and glycine were determined in all of the samples assayed at the latter institution. It was established that the sample preservation processes and the microbiological procedures employed were adequate. The distribution of amino acids in the canned fish and meat products was generally uniform. None of the 13 amino acids determined was significantly altered by the heat processing to which the canned samples had been subjected. Data from both laboratories are presented and discussed in the second part of this report (Neilands et al., '49).

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# THE NUTRITIVE VALUE OF CANNED FOODS[1]

## II. AMINO ACID CONTENT OF FISH AND MEAT PRODUCTS

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This is the second portion of the report of an inter-laboratory amino acid assay project involving the University of Wisconsin and the University of California at Los Angeles. An introduction to the project, a consideration of the purpose of the work, and a description of the samples analyzed can be found in the immediately preceding paper (part I).

In our laboratory a total of 61 individual samples were assayed for the 10 essential amino acids. Of this total, 41 were identical samples assayed also at U.C.L.A. by Dunn et al. ('49). In the present paper the assay results for the samples assayed in both laboratories will be presented and discussed. Results for the 20 additional samples assayed in this laboratory only will also be presented.

A proximate analysis is given for each sample in order that the amino acid content may be calculated on any desired basis.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. The authors are indebted to Miss Martha Johnson for technical assistance with a part of this project. This work was a part of the National Canners' Association — Can Manufacturers' Institute nutrition program, and is No. XXXIII in the series on the nutritive value of canned foods.

## EXPERIMENTAL

### *Samples*

Details of the exact nature, manner of collection and distribution of the samples are given in the preceding paper (part I). For convenience each sample was assigned a code designation made up of two initials indicating the type of product, a number to distinguish among multiple samples of the same type of product put up at different canneries and, finally, the letters R, RC or P denoting raw, pre-cooked or heat-processed, respectively. The initial letters used for each type of product were as follows: MA, Atlantic mackerel; MP, Pacific mackerel; SO, Atlantic sardines in oil; ST, Pacific sardines in tomato sauce; FA, Atlantic fish flakes; SP, shrimp; SC, salmon; SH, spiced ham; WH, whole ham; TO, tuna in oil; RB, beef. All the different foodstuffs investigated, with the exception of shrimp, were represented both by processed samples and by a certain number of raw companion samples. The raw samples were received in a frozen state and were held at — 4°C. until used. The heat-processed samples were allowed to remain at room temperature in the original sealed containers.

In almost every instance the entire product, including solid and liquid portions of the canned foods, was homogenized by being passed through a commercial meat grinder several times. The three shrimp samples were handled differently, since only the solid portion of the pack was sampled.[2] Immediately after homogenizing, samples were weighed out for hydrolysis and total N determinations. The product was then placed in a tightly closed screw-cap jar and held in the deep freeze (—4°C.) until aliquots for dry weight, ash and fat analyses had been taken.

To prepare each sample for tryptophan assay a 5-gm aliquot was placed in a stainless steel beaker and hydrolyzed in 5 N NaOH at 121°C. for 15 hours. This was assumed to race-

[2] At U.C.L.A. both the solid and liquid portions were assayed. These three shrimp samples, therefore, are reported as separate samples assayed at each university (cf. Dunn et al., '49, footnote 3).

mize completely the tryptophan present. The hydrolysate was acidified with excess acetic acid and extracted three times with ether to remove the fat. If glass rather than stainless steel or nickel beakers were used for this hydrolysis a voluminous precipitate, presumably silica, was always obtained on acidification. For the assay of the 9 remaining amino acids the hydrolysate was prepared on a 5-gm aliquot with 3 N HCl at 121°C. for 5 hours.

### *Assay methods*

In our laboratory the proximate analyses (table 1) were carried out as follows: Per cent moisture in the sample was found by drying a 5-gm aliquot to constant weight (usually 48 hrs.) at 100°C., followed by drying overnight over $CaCl_2$. Ash was found by igniting the dry residue from the moisture determination at 800°C. to constant weight (about 6 hrs.). Protein was calculated from total N $\times$ 6.25 where nitrogen was determined by the macro Kjeldahl method. Since the amino acid content of the samples was calculated on the basis of per cent of crude protein, both the Wisconsin and U.C.L.A. protein values are reported for each common sample.

The uniform medium of Henderson and Snell ('48) and the techniques described by these workers were used for all of the amino acid determinations except in analyses where *Leuconostoc citrovorum* 8081 was used as the assay organism. In the latter assays the medium was supplemented with liver concentrate [3] at the level suggested in the recent paper of Steele et al. ('49).

The amino acids used as standards possessed microbiological activity that compared well, within the limits of experimental error, with standards supplied to 20 laboratories for the comparative study of 6 proteins in a project coordinated by Dr. W. H. Cole, Bureau of Biological Research, Rutgers University. A sample of DL-isoleucine supplied by Rutgers University was used as the standard, since other available

[3] Reticulogen, Lilly.

TABLE 1

*Proximate analysis of fish and meat samples* [1]

| CODE [2] | MOISTURE | FAT | ASH | PROTEIN | |
|---|---|---|---|---|---|
| | | | | A [3] | B [4] |
| | % | % | % | % | % |
| MA1R | 65.1 | 16.3 | 2.92 | 17.8 | 17.4 |
| MA1P | 61.7 | 18.0 | 2.80 | 18.0 | 17.6 |
| MA2R | 61.1 | 19.0 | 3.01 | 17.7 | 17.3 |
| MA2P | 61.3 | 16.7 | 2.84 | 18.7 | 17.8 |
| MA3P | 61.7 | 17.6 | 3.23 | 17.7 | ... |
| MA4P | 69.0 | 5.84 | 3.59 | 19.1 | ... |
| MP1P | 68.1 | 5.47 | 2.20 | 26.0 | ... |
| MP2P | 62.5 | 8.22 | 2.19 | 22.1 | ... |
| MP5R | 71.5 | 3.12 | 2.40 | 21.4 | 22.8 |
| MP5P | 73.7 | 3.13 | 2.83 | 24.9 | 20.7 |
| MP6R | 73.1 | 2.36 | 2.43 | 20.0 | 20.7 |
| MP6P | 70.5 | 5.49 | 2.94 | 23.1 | 21.9 |
| SR [4] | 70.9 | 5.08 | 1.87 | 20.9 | 20.6 |
| SO1R | 59.5 | 17.8 | 2.42 | 21.3 | 20.2 |
| SO1P | 60.9 | 15.2 | 2.42 | 20.9 | ... |
| SO2R | 68.8 | 9.95 | 2.71 | 21.4 | 21.3 |
| SO2P | 66.8 | 9.87 | 2.87 | 21.8 | 17.9 |
| SO3P | 57.8 | 17.3 | 4.52 | 20.4 | ... |
| SO4P | 53.4 | 17.8 | 4.05 | 23.5 | ... |
| ST1P | 67.0 | 10.4 | 2.57 | 18.6 | ... |
| ST2P | 63.5 | 10.3 | 2.57 | 18.8 | ... |
| ST5R | 68.2 | 10.7 | 2.87 | 18.8 | 17.1 |
| ST5P | 67.5 | 10.4 | 3.07 | 17.3 | 18.0 |
| ST6R | 69.9 | 9.08 | 2.42 | 17.5 | 16.8 |
| ST6RC | 67.4 | 10.5 | 2.69 | 19.3 | 17.1 |
| ST6P | 66.5 | 10.3 | 2.91 | 20.5 | 19.3 |
| FA1R | 72.4 | 0.22 | 2.16 | 24.0 | 25.0 |
| FA1P | 68.2 | 0.31 | 3.18 | 24.5 | 25.3 |
| FA2R | 72.9 | 0.24 | 1.97 | 22.8 | 25.6 |
| FA2P | 70.7 | 0.27 | 2.45 | 23.0 | 25.0 |
| FA3P | 68.8 | 0.43 | 2.61 | 24.6 | ... |
| FA4P | 67.3 | 0.53 | 2.43 | 21.0 | ... |
| SP1P | 69.9 | 0.63 | 3.91 | 26.8 | ... |
| SP2P | 70.1 | 0.75 | 2.85 | 21.4 | ... |
| SP3P | 72.3 | 0.86 | 4.23 | 21.8 | ... |
| SC1R | 59.0 | 14.3 | 2.97 | 20.2 | 18.3 |
| SC1P | 59.4 | 17.5 | 2.24 | 19.0 | 18.3 |
| SC2R | 65.4 | 10.4 | 2.16 | 20.2 | 20.4 |
| SC2P | 64.7 | 9.60 | 2.81 | 22.7 | 19.1 |
| SC4P | 54.6 | 24.7 | 1.41 | 18.5 | ... |
| SC6P | 53.6 | 25.4 | 1.18 | 18.5 | ... |

TABLE 1 (*continued*)

| CODE [2] | MOISTURE | FAT | ASH | PROTEIN | |
|---|---|---|---|---|---|
| | | | | A [3] | B [3] |
| | % | % | % | % | % |
| SH1R | 54.9 | 23.8 | 1.82 | 14.4 | 14.8 |
| SH1P | 56.0 | 24.0 | 2.94 | 14.6 | 15.8 |
| SH4P | 53.1 | 24.1 | 4.39 | 15.3 | ... |
| SH5P | 54.3 | 23.7 | 4.36 | 15.1 | ... |
| SH6R | 54.0 | 28.6 | 3.87 | 13.1 | 13.1 |
| SH6P | 52.8 | 26.6 | 2.40 | 13.8 | 13.6 |
| WH1R | 60.9 | 17.5 | 1.62 | 15.9 | 15.4 |
| WH1P | 58.8 | 19.2 | 2.25 | 16.0 | 14.8 |
| WH2R | 60.3 | 19.5 | 3.61 | 15.5 | 16.0 |
| WH2P | 56.6 | 23.9 | 2.06 | 17.1 | 16.5 |
| TO1P | 49.2 | 22.3 | 2.06 | 25.6 | ... |
| TO3R | 70.9 | 0.19 | 1.53 | 25.4 | 25.8 |
| TO3RC | 50.4 | 17.4 | 2.42 | 25.6 | 25.0 |
| TO3P | 50.4 | 23.4 | 1.53 | 25.5 | 25.0 |
| TO5R | 63.6 | 11.8 | 1.42 | 25.5 | 26.6 |
| TO5RC | 52.7 | 18.9 | 2.82 | 26.4 | 29.2 |
| TO5P | 51.1 | 20.9 | 2.62 | 25.5 | 25.6 |
| TO6P | 57.2 | 13.4 | 3.14 | 27.7 | ... |
| RB1R | 71.9 | 4.06 | 1.21 | 21.4 | 20.0 |
| RB1P | 68.8 | 6.88 | 1.08 | 22.7 | 21.5 |

[1] Values for fat represent the results of a single determination carried out by the Wisconsin Alumni Research Foundation; those for ash are averages of values obtained at Wisconsin and U.C.L.A., while those for moisture are averages of values obtained at Wisconsin, U.C.L.A., and the above Foundation laboratories.

[2] A complete interpretation of the sample code number is given in the text.

[3] A — analyses done at Wisconsin.
B — analyses done at U.C.L.A.

[4] A sample of raw Pacific sardines.

lots of isoleucine appeared to be contaminated, perhaps with alloisoleucine. L-methionine [4] was used for the methionine analyses because previous work in this laboratory had indicated that the D-isomer of DL-methionine had partial activity for *Streptococcus faecalis* R under our assay conditions.

Assays were prepared for simultaneous analyses with two or more organisms wherever this was possible. Accordingly, for each of the amino acids determined at least two of the

[4] We wish to thank Dr. David Doherty for this sample of pure, enzymatically resolved L-methionine.

organisms indicated were used except in the case of lysine and threonine, for which assays only the single organisms are suitable. The different cultures used and the amino acids assayed by each were as follows:

| | | |
|---|---|---|
| Arginine | *L. delbrueckii* 3 | *L. citrovorum* 8081 |
| Histidine | *L. delbrueckii* 3 | *L. mesenteroides* P-60 |
| Isoleucine | *L. delbrueckii* 3 | *L. mesenteroides* P-60 |
| Leucine | *L. delbrueckii* 3 | *L. arabinosus* 17–5 |
| Lysine | *L. mesenteroides* P-60 | |
| Methionine | *L. mesenteroides* P-60 | *S. faecalis* R |
| | *L. citrovorum* 8081 | *L. fermenti* 36 |
| Phenylalanine | *L. arabinosus* 17–5 | *L. delbrueckii* 3 |
| Threonine | *S. faecalis* R | |
| Tryptophan | *S. faecalis* R | *L. arabinosus* 17–5 |
| Valine | *L. delbrueckii* 3 | *L. arabinosus* 17–5 |

The results were averaged when more than one organism was used for the determination of a given amino acid. This was the case for 380 of the 610 individual values from this laboratory given in table 2.

Titration of the acid formed by the assay organisms was carried out after three days at 37°C. All the analyses done in this laboratory and reported in table 2 were performed on duplicate aliquots of the original sample weighed out separately and hydrolyzed.

To facilitate discussion of the results, table 3 was prepared. It contains an average of the amino acid content of each type of product, both raw and processed, reported in table 2.

## DISCUSSION

### *Essential amino acid composition of fish and meat products*

All amino acid values reported and discussed in this paper will be in terms of per cent of crude protein; that is, in grams of amino acid per 16 gm total N.

An inspection of table 3 at once reveals a striking similarity, already noted by Block and Bolling ('45), in the amino acid composition of the various products analyzed. In general, lysine, methionine and tryptophan are the three essential

TABLE 2

*Amino acid content of fish and meat products*

(Values given as per cent of protein, N × 6.25)

| CODE [1] | MA1R | | MA1P | | MA2R | | MA2P | | MA3P | MA4P | MP1P | MP2P | MP5R | | MP5P | | MP6R | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMINO ACID | A [2] | B [2] | A | B | A | B | A | B | A | A | A | A | A | B | A | B | A | B |
| Arginine | 5.6 | 5.6 | 5.8 | 5.8 | 5.7 | 5.7 | 5.7 | 5.7 | 6.1 | 6.1 | 5.1 | 5.5 | 6.1 | 5.2 | 5.5 | 5.4 | 5.4 | 5.2 |
| Histidine | 4.2 | 4.1 | 3.7 | 3.7 | 3.8 | 3.9 | 3.5 | 3.7 | 3.7 | 3.8 | 5.0 | 4.8 | 5.9 | 4.6 | 4.9 | 5.2 | 5.6 | 6.0 |
| Isoleucine | 5.5 | 5.0 | 5.5 | 4.8 | 5.6 | 5.0 | 5.6 | 4.5 | 5.8 | 4.7 | 4.1 | 4.7 | 5.0 | 6.4 | 4.3 | 5.0 | 4.7 | 5.0 |
| Leucine | 6.9 | 7.0 | 7.2 | 7.4 | 7.0 | 7.1 | 6.9 | 7.1 | 7.6 | 7.5 | 6.6 | 7.6 | 7.6 | 7.8 | 7.1 | 7.9 | 6.7 | 8.1 |
| Lysine | 8.0 | 8.6 | 7.7 | 9.0 | 7.7 | 8.4 | 7.6 | 8.7 | 8.0 | 7.4 | 9.3 | 7.0 | 9.6 | 8.5 | 8.6 | 8.0 | 8.7 | 8.3 |
| Methionine | 2.6 | 2.8 | 2.6 | 2.8 | 2.6 | 2.8 | 2.5 | 2.9 | 2.5 | 3.2 | 2.8 | 2.8 | 2.8 | 2.7 | 2.9 | 2.8 | 2.8 | 2.8 |
| Phenylalanine | 3.3 | 3.8 | 3.6 | 3.6 | 3.3 | 3.7 | 3.4 | 3.4 | 3.7 | 3.3 | 3.6 | 3.8 | 4.1 | 4.0 | 3.7 | 3.9 | 3.7 | 3.9 |
| Threonine | 4.6 | 5.5 | 4.7 | 6.3 | 4.4 | 4.4 | 4.4 | 5.8 | 4.5 | 4.0 | 4.2 | 5.1 | 4.4 | 4.2 | 4.1 | 4.6 | 4.5 | 4.6 |
| Tryptophan | 0.9 | 0.9 | 0.9 | 0.3 [3] | 0.9 | 0.9 | 0.9 | 0.3 [3] | 0.9 | 1.0 | 1.0 | 0.9 | 1.1 | 1.0 | 1.0 | 1.0 | 1.1 | 1.0 |
| Valine | 5.6 | 5.7 | 5.2 | 5.4 | 5.6 | 5.5 | 5.4 | 5.1 | 5.4 | 5.2 | 4.6 | 5.1 | 5.5 | 5.5 | 5.0 | 5.6 | 4.8 | 5.6 |

| CODE [1] | MP6P | | SR [4] | | SO1R | | SO1P | SO2R | | SO2P | | SO3P | SO4P | ST1P | ST2P | ST5R | | ST5P | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMINO ACID | A | B | A | B | A | B | A | A | B | A | B | A | A | A | A | A | B | A | B |
| Arginine | 5.6 | 5.6 | 4.8 | 5.0 | 5.9 | 5.4 | 5.6 | 5.9 | 4.8 | 6.1 | 5.5 | 5.8 | 4.9 | 5.3 | 5.1 | 4.9 | 6.0 | 5.2 | 4.5 |
| Histidine | 5.2 | 5.3 | 4.9 | 5.0 | 2.5 | 2.6 | 2.4 | 2.5 | 2.3 | 2.3 | 2.4 | 2.1 | 2.2 | 4.4 | 4.5 | 4.6 | 5.5 | 5.0 | 4.3 |
| Isoleucine | 4.9 | 5.4 | 3.6 | 5.0 | 5.4 | 4.8 | 5.3 | 5.3 | 3.8 | 5.3 | 4.5 | 5.2 | 4.1 | 4.4 | 4.3 | 4.2 | 5.2 | 4.5 | 4.3 |
| Leucine | 6.9 | 7.4 | 6.3 | 6.0 | 7.2 | 7.2 | 7.2 | 7.1 | 6.2 | 7.3 | 7.4 | 7.1 | 7.4 | 7.2 | 7.1 | 6.5 | 8.2 | 7.3 | 7.3 |
| Lysine | 9.1 | 8.2 | 8.0 | 8.5 | 7.8 | 8.4 | 7.8 | 7.6 | 7.5 | 7.5 | 8.9 | 7.9 | 7.0 | 6.8 | 8.0 | 8.6 | 9.0 | 9.7 | 7.2 |
| Methionine | 2.9 | 2.7 | 2.5 | 2.7 | 2.8 | 3.0 | 2.5 | 2.5 | 2.6 | 2.7 | 3.0 | 2.5 | 2.9 | 3.0 | 2.8 | 2.7 | 2.7 | 2.9 | 2.4 |
| Phenylalanine | 3.8 | 3.9 | 3.2 | 3.7 | 3.5 | 3.0 | 3.6 | 3.5 | 3.2 | 3.5 | 3.7 | 3.5 | 3.7 | 3.6 | 3.6 | 3.8 | 4.2 | 3.7 | 3.4 |
| Threonine | 4.3 | 4.7 | 3.8 | 4.2 | 4.2 | 3.8 | 4.2 | 4.3 | 4.8 | 3.9 | 5.3 | 4.2 | 4.6 | 4.6 | 4.6 | 4.3 | 4.6 | 4.2 | 3.6 |
| Tryptophan | 1.2 | 1.0 | 1.1 | 1.1 | 0.8 | 0.8 | 0.8 | 0.8 | 0.7 | 0.7 | 0.8 | 0.7 | 0.8 | 0.8 | 0.9 | 0.9 | 1.0 | 1.0 | 1.1 |
| Valine | 4.7 | 5.5 | 4.3 | 5.4 | 5.2 | 5.4 | 5.4 | 5.2 | 4.2 | 5.2 | 5.2 | 4.9 | 4.7 | 4.8 | 4.7 | 4.8 | 5.6 | 5.0 | 5.3 |

TABLE 2 (*continued*)

| CODE [1] | ST6R | | ST6RC | | ST6P | | FA1R | | FA1P | | FA2R | | FA2P | | FA3P | FA4P | SP1P | SP2P |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMINO ACID | A | B | A | B | A | B | A | B | A | B | A | B | A | B | A | A | A | A |
| Arginine | 5.0 | 4.6 | 5.4 | 5.4 | 5.0 | 4.6 | 6.1 | 6.1 | 6.2 | 5.8 | 6.3 | 5.5 | 6.2 | 6.4 | 6.2 | 6.6 | 8.6 | 10.4 |
| Histidine | 4.5 | 4.7 | 4.6 | 5.1 | 4.3 | 4.2 | 2.1 | 2.1 | 2.1 | 2.1 | 1.9 | 1.8 | 2.0 | 2.0 | 2.1 | 2.5 | 2.1 | 2.3 |
| Isoleucine | 4.3 | 5.4 | 4.5 | 5.3 | 4.3 | 4.6 | 6.1 | 5.2 | 6.3 | 4.7 | 6.1 | 4.6 | 6.1 | 5.2 | 6.1 | 5.0 | 5.0 | 5.8 |
| Leucine | 6.8 | 7.9 | 7.3 | 8.6 | 7.0 | 7.5 | 8.3 | 8.2 | 8.2 | 7.8 | 8.1 | 7.2 | 8.0 | 8.4 | 7.9 | 8.4 | 8.0 | 9.6 |
| Lysine | 8.9 | 8.0 | 9.5 | 9.4 | 8.4 | 7.8 | 8.5 | 10.0 | 8.7 | 9.4 | 8.7 | 8.7 | 8.4 | 9.8 | 8.9 | 7.3 | 8.6 | 9.0 |
| Methionine | 2.8 | 2.6 | 3.0 | 2.9 | 2.9 | 2.8 | 2.7 | 3.4 | 2.6 | 3.2 | 3.0 | 3.0 | 2.7 | 3.5 | 2.7 | 3.3 | 3.2 | 3.7 |
| Phenylalanine | 3.8 | 3.6 | 3.8 | 4.2 | 3.7 | 3.8 | 3.8 | 3.9 | 3.7 | 3.7 | 3.8 | 3.5 | 3.8 | 4.1 | 3.9 | 3.6 | 4.0 | 5.0 |
| Threonine | 4.2 | 4.6 | 4.6 | 5.2 | 4.0 | 4.2 | 4.4 | 6.0 | 4.3 | 5.8 | 4.5 | 5.2 | 4.5 | 6.0 | 4.4 | 4.3 | 4.3 | 4.2 |
| Tryptophan | 1.0 | 1.0 | 1.1 | 1.0 | 1.1 | 1.0 | 1.1 | 1.1 | 1.0 | 1.0 | 1.0 | 0.9 | 1.0 | 0.9 | 1.0 | 1.1 | 1.0 | 1.1 |
| Valine | 4.7 | 5.7 | 5.1 | 6.0 | 5.1 | 5.8 | 5.6 | 5.4 | 5.5 | 5.1 | 5.3 | 4.7 | 5.5 | 5.4 | 5.6 | 5.1 | 5.1 | 5.3 |

| CODE [1] | SP3P | SC1R | | SC1P | | SC2R | | SC2P | | SC4P | SC6P | SH1R | | SH1P | | SH4P | SH5P | SH6R | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMINO ACID | A | A | B | A | B | A | B | A | B | A | A | A | B | A | B | A | A | A | B |
| Arginine | 9.1 | 5.8 | 5.5 | 5.9 | 5.5 | 5.7 | 5.8 | 5.6 | 5.4 | 6.0 | 6.3 | 6.2 | 6.2 | 6.3 | 5.4 | 6.4 | 6.4 | 6.3 | 6.4 |
| Histidine | 2.1 | 2.7 | 2.7 | 2.7 | 2.7 | 2.6 | 2.4 | 2.5 | 2.3 | 2.5 | 2.6 | 2.8 | 2.9 | 2.8 | 2.7 | 3.5 | 3.5 | 2.8 | 3.3 |
| Isoleucine | 5.1 | 4.7 | 5.4 | 4.9 | 5.0 | 4.6 | 5.2 | 4.6 | 4.9 | 4.8 | 4.8 | 4.3 | 4.5 | 4.4 | 4.4 | 4.7 | 4.6 | 4.4 | 4.9 |
| Leucine | 7.9 | 6.8 | 7.2 | 7.2 | 7.6 | 7.0 | 7.5 | 6.8 | 7.3 | 7.5 | 7.7 | 7.4 | 7.6 | 8.2 | 6.7 | 7.9 | 8.3 | 7.9 | 8.0 |
| Lysine | 7.9 | 7.5 | 8.4 | 8.0 | 8.5 | 7.8 | 8.8 | 7.6 | 8.3 | 7.5 | 7.9 | 8.2 | 8.9 | 7.9 | 7.6 | 7.7 | 7.8 | 7.7 | 8.9 |
| Methionine | 3.3 | 3.0 | 2.8 | 3.1 | 3.0 | 3.1 | 3.0 | 2.9 | 2.8 | 3.5 | 3.2 | 2.4 | 2.4 | 2.4 | 2.0 | 2.7 | 2.6 | 2.5 | 2.4 |
| Phenylalanine | 4.4 | 3.4 | 3.7 | 3.7 | 3.7 | 3.7 | 3.8 | 3.5 | 3.5 | 3.6 | 4.0 | 3.6 | 3.9 | 3.7 | 3.5 | 3.6 | 3.9 | 3.8 | 4.1 |
| Threonine | 3.9 | 4.0 | 4.0 | 4.6 | 4.6 | 4.4 | 4.4 | 4.7 | 4.4 | 4.3 | 4.4 | 4.9 | 3.6 | 4.9 | 2.9 | 4.6 | 4.7 | 4.3 | 4.4 |
| Tryptophan | 0.8 | 0.9 | 0.9 | 1.0 | 0.8 | 1.0 | 0.9 | 0.9 | 0.4 [2] | 1.0 | 1.0 | 0.9 | 0.9 | 1.0 | 0.8 | 0.9 | 0.9 | 0.9 | 0.9 |
| Valine | 5.0 | 5.3 | 5.8 | 5.4 | 5.9 | 5.3 | 5.7 | 5.2 | 5.6 | 5.8 | 5.6 | 4.8 | 5.0 | 4.9 | 4.6 | 4.9 | 4.9 | 4.9 | 5.4 |

TABLE 2 (*continued*)

| CODE [1] | SH6P | | WH1R | | WH1P | | WH2R | | WH2P | | TO1P | TO3R | | TO3RC | | TO3P | | TO5R | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AMINO ACID | A | B | A | B | A | B | A | B | A | B | A | A | B | A | B | A | B | A | B |
| Arginine | 6.3 | 6.3 | 6.1 | 6.1 | 6.3 | 6.2 | 6.4 | 6.7 | 6.9 | 6.3 | 5.0 | 5.3 | 5.2 | 5.1 | 5.3 | 5.0 | 5.3 | 5.7 | 5.2 |
| Histidine | 3.2 | 3.2 | 3.7 | 3.6 | 3.4 | 3.5 | 3.3 | 3.6 | 3.5 | 3.3 | 5.8 | 5.6 | 5.7 | 6.0 | 5.8 | 5.7 | 6.0 | 6.0 | 5.6 |
| Isoleucine | 4.6 | 4.9 | 4.5 | 5.1 | 4.7 | 4.8 | 4.6 | 5.3 | 4.8 | 5.2 | 4.1 | 3.8 | 4.7 | 4.1 | 5.0 | 4.0 | 4.9 | 4.8 | 4.4 |
| Leucine | 7.7 | 7.7 | 7.4 | 8.1 | 7.9 | 7.6 | 7.6 | 8.3 | 8.2 | 7.8 | 7.0 | 6.6 | 7.0 | 6.9 | 7.3 | 6.8 | 7.3 | 7.2 | 6.9 |
| Lysine | 7.9 | 8.5 | 8.2 | 9.1 | 9.1 | 8.8 | 8.3 | 9.9 | 8.7 | 8.9 | 8.6 | 7.7 | 8.3 | 8.7 | 8.7 | 7.6 | 8.7 | 8.3 | 8.6 |
| Methionine | 2.5 | 2.3 | 2.4 | 2.4 | 2.5 | 2.4 | 2.7 | 2.8 | 2.6 | 2.5 | 2.7 | 2.7 | 2.8 | 2.7 | 2.9 | 2.7 | 2.8 | 3.0 | 2.8 |
| Phenylalanine | 3.5 | 3.8 | 3.3 | 4.1 | 3.7 | 4.0 | 3.5 | 4.3 | 3.4 | 4.0 | 3.3 | 3.0 | 3.5 | 3.3 | 3.6 | 3.1 | 3.7 | 3.6 | 3.4 |
| Threonine | 4.3 | 4.3 | 4.1 | 4.2 | 4.8 | 3.5 | 4.6 | 3.9 | 4.7 | 3.6 | 3.8 | 4.0 | 4.1 | 4.4 | 4.7 | 4.3 | 4.4 | 4.5 | 6.3 |
| Tryptophan | 0.8 | 0.7 | 1.0 | 1.0 | 0.9 | 0.8 | 1.1 | 1.0 | 1.1 | 1.0 | 1.0 | 1.1 | 1.1 | 1.1 | 1.0 | 1.0 | 0.7 | 1.0 | 0.4 [3] |
| Valine | 4.8 | 5.2 | 5.1 | 5.5 | 5.4 | 5.3 | 5.2 | 5.7 | 5.3 | 5.4 | 4.4 | 4.3 | 5.2 | 4.5 | 5.5 | 4.3 | 5.4 | 5.4 | 5.1 |

| CODE [1] | TO5RC | | TO5P | | TO6P | RB1R | | RB1P | |
|---|---|---|---|---|---|---|---|---|---|
| AMINO ACID | A | B | A | B | A | A | B | A | B |
| Arginine | 5.6 | 4.6 | 5.6 | 5.0 | 5.6 | 5.8 | 6.3 | 6.1 | 6.0 |
| Histidine | 5.4 | 5.1 | 5.6 | 5.2 | 6.6 | 3.8 | 3.7 | 3.6 | 3.4 |
| Isoleucine | 5.1 | 4.8 | 5.3 | 5.4 | 5.3 | 4.9 | 5.5 | 4.7 | 5.0 |
| Leucine | 7.2 | 7.2 | 7.5 | 7.2 | 7.9 | 7.6 | 8.3 | 7.4 | 7.7 |
| Lysine | 8.0 | 8.1 | 8.5 | 8.1 | 8.3 | 8.1 | 9.7 | 8.0 | 8.8 |
| Methionine | 2.9 | 2.7 | 2.9 | 2.6 | 3.1 | 2.7 | 2.7 | 2.7 | 2.5 |
| Phenylalanine | 3.8 | 3.3 | 4.0 | 3.7 | 4.1 | 3.8 | 4.1 | 3.4 | 3.7 |
| Threonine | 4.7 | 4.5 | 4.7 | 4.5 | 4.0 | 4.4 | 4.9 | 4.5 | 4.3 |
| Tryptophan | 1.0 | 0.4 [3] | 1.0 | 0.9 | 1.0 | 1.0 | 1.1 | 0.9 | 0.9 |
| Valine | 5.5 | 5.2 | 5.6 | 5.7 | 5.8 | 5.2 | 5.5 | 4.9 | 5.0 |

[1] A complete explanation of the sample code is given in the text.

[2] A — assays done at Wisconsin.
B — assays done at U.C.L.A.

[3] These values appear to be out of line. The reason for this is unknown.

[4] A sample of raw Pacific sardines.

TABLE 3

*Average amino acid content of fish and meat samples* [1]

(Values given as per cent of protein, N × 6.25)

| NO. OF SAMPLES | ATLANTIC MACKEREL 6 | PACIFIC MACKEREL 6 | ATLANTIC SARDINES 6 | PACIFIC SARDINES 8 | FISH FLAKES 6 | SHRIMP 3 | SALMON 6 | SPICED HAM 6 | WHOLE HAM 4 | TUNA 8 | BEEF 2 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Amino acid* | | | | | | | | | | | |
| Arginine | 5.8 | 5.5 | 5.5 | 5.1 | 6.1 | 9.4 | 5.8 | 6.2 | 6.4 | 5.3 | 6.1 |
| Histidine | 3.8 | 5.4 | 2.4 | 4.7 | 2.1 | 2.2 | 2.6 | 3.1 | 3.5 | 5.7 | 3.6 |
| Isoleucine | 5.2 | 5.0 | 4.9 | 4.6 | 5.5 | 5.3 | 4.9 | 4.6 | 4.9 | 4.7 | 5.0 |
| Leucine | 7.2 | 7.4 | 7.1 | 7.2 | 8.1 | 8.5 | 7.3 | 7.7 | 7.9 | 7.2 | 7.8 |
| Lysine | 8.1 | 8.5 | 7.8 | 8.4 | 8.8 | 8.5 | 8.0 | 8.1 | 8.9 | 8.3 | 8.7 |
| Methionine | 2.7 | 2.8 | 2.7 | 2.8 | 3.0 | 3.4 | 3.0 | 2.4 | 2.5 | 2.8 | 2.7 |
| Phenylalanine | 3.5 | 3.8 | 3.4 | 3.7 | 3.9 | 4.5 | 3.7 | 3.8 | 3.8 | 3.5 | 3.8 |
| Threonine | 4.9 | 4.5 | 4.4 | 4.3 | 4.9 | 4.1 | 4.4 | 4.3 | 4.2 | 4.5 | 4.5 |
| Tryptophan | 1.0 | 1.0 | 0.8 | 1.0 | 1.0 | 1.0 | 0.9 | 0.9 | 1.0 | 1.0 | 1.0 |
| Valine | 5.4 | 5.2 | 5.0 | 5.2 | 5.3 | 5.1 | 5.6 | 4.9 | 5.4 | 5.1 | 5.2 |

[1] Average of all values in table 2 except those marked and indicated to be out of line.

amino acids most important in evaluating the nutritive quality of a protein. A deficiency of these amino acids is frequently encountered in natural food materials. All the products tested in this survey are apparently very good sources of lysine and good sources of tryptophan and methionine and the other essential amino acids. These products must be considered to contain protein of the finest nutritive quality.

In marked contrast to the very small variation found for each specific amino acid in *different* products, we record one exception, i.e., histidine. Commenting upon a similar observation, Lyman states in a recent publication (Lyman and Kuiken, '49) ". . . variation in the histidine content of muscle tissue is due to the presence of substantial amounts of derivatives of histidine which are not part of the true protein of the tissue. These histidine derivatives are carnosine, a peptide of beta-alanine and histidine, and anserine, a peptide of beta-alanine and methyl histidine. A study of the relationship of these compounds to the histidine content of meat has been reported by Lyman, Kuiken and Hale ('47). As much as one-third of the total histidine content of beef loin or other beef muscle tissue may be in the form of carnosine."

The existing literature contains relatively little information on the amino acid content of the edible portions of various fish, and even where data are available comparisons are often difficult because the fish analyzed have been inadequately identified. In the following discussion the methods used for determining the amino acids in question are indicated in parentheses. Further details regarding the indicated methods may be found in Block and Bolling ('45).

Pottinger and Baldwin ('39) have investigated the arginine, histidine, lysine (Block) and tryptophan (Folin and Ciocalteau) content of a series of fishery products. Although only 4 amino acids were determined, the materials analyzed were identified by their scientific names and therefore we believe that our results can be compared with confidence to those of Pottinger and Baldwin.

Atlantic mackerel assayed by these workers was found to contain 5.8% arginine, 1.9% histidine, and 7.1% lysine. Our analysis of this species (table 3) shows 5.8% arginine, 3.8% histidine and 8.1% lysine. Pottinger and Baldwin mention that since their methods depend on the isolation of the amino acids as a salt, their results must be regarded as minimum. The tryptophan content was found by them to be 1.4% as compared to 0.98% in our analysis.

The same authors found that mature Atlantic herring contained 5.1% arginine, 1.6% histidine, 7.0% lysine and 1.2% tryptophan, as contrasted to 5.5, 2.4, 7.8 and 0.77%, respectively, for our samples.

Pilchards (*Sardinia pilchardii*) contained 5.6% arginine, 1.2% histidine, 6.8% lysine and 1.3% tryptophan, while our Pacific sardines (*Sardinia caerulea*), probably a very closely related species, contained 5.1% arginine, 4.7% histidine, 8.5% lysine and 0.98% tryptophan.

Cod and haddock analyzed by Pottinger and Baldwin and the Atlantic fish flakes assayed by the present authors contained, respectively, 5.6, 5.7 and 6.1% arginine; 1.7, 1.2 and 2.1% histidine; 6.8, 6.4 and 8.8% lysine; and 1.06, 0.85 and 0.99% tryptophan.

It is noteworthy that the products assayed by chemical methods as contrasted with the newer microbiological assays were consistently very similar for arginine content, somewhat lower for lysine, higher for tryptophan and much lower for histidine. Fuller et al. ('47) believe that the carnosine and hence the histidine content of muscle tissue may vary with the amount of dietary histidine.

Cod, analyzed by Beach et al. ('43), and the Atlantic fish flakes of this survey contained, respectively, 6.3% (Block) and 6.1% arginine; 2.0% (Block) and 2.1% histidine; 8.4% (Block) and 8.8% lysine; 3.7% (Sullivan) and 3.0% methionine; 4.3% (Block) and 3.9% phenylalanine and 1.3% (Lugg) and 0.99% tryptophan. Again the microbiological method gave a lower tryptophan content. The other analyses are in fairly good agreement.

Edwards et al. ('46) determined the essential amino acids in fresh haddock. They found 9.2% arginine (Weber-Bartlett), compared to 6.1% arginine for our Atlantic fish flakes. The values for phenylalanine (Block) and tryptophan (Lugg, Brand and Kassell) were very close to those found by the present authors using microbiological methods.

Shrimp has been rather thoroughly assayed for amino acid content. Beach et al. ('43) obtained remarkably similar figures to those in table 3 for histidine (Block), lysine (Block), methionine (Sullivan), phenylalanine (Block) and tryptophan (Lugg). They found an arginine content of 6.6% (Block), compared to 9.4% in our samples. The shrimp sample analyzed by Pottinger and Baldwin ('39) contained 7.5% arginine (Block) and was considerably higher in this amino acid than the true fish products assayed. Possibly the phosphoarginine (Baldwin, '47) of invertebrate muscle may account for the higher arginine values found in these instances.

Employing chemical methods to examine salmon for 5 of the essential amino acids, Beach et al. ('43) obtained results in fair agreement with those reported for salmon in table 3. Deas and Tarr ('49) have investigated the amino acid content of various fishery by-products using the microbiological assay.

In contrast to the scarcity of data for the amino acid composition of fish, the literature affords relatively ample data for various types of meat samples. Table 4 was drawn up to compare our values for pork and meat with those in the literature. This table again illustrates the similarity of the essential amino acid composition of animal proteins. Differences between our averages and those in the literature are probably caused primarily by inherent fluctuations in the assay method, plus the effect of abnormally high or low figures on the literature average.

### *Reliability of the microbiological assay*

In reviewing the literature of amino acid analysis the authors were impressed by the oft-repeated statement that the chemical determination of only a few of the essential

amino acids in a relatively small number of products could develop into a formidable task. The microbiological assay eliminates much of the tedious work required by chemical methods, and renders more extensive surveys feasible. However, the newness of the microbiological methods makes more data on their reliability highly desirable, and the present study provides some information on this question.

TABLE 4

*Comparison of the present data with values in the literature for the essential amino acid composition of pork and beef*

(Values given as per cent of protein, N × 6.25)

| AMINO ACID | PORK MUSCLE | | | | BEEF MUSCLE | | | |
|---|---|---|---|---|---|---|---|---|
| | Present [1] project | Literature [2] | | | Present [1] project | Literature [2] | | |
| | | Ave. | Range | No. of analyses | | Ave. | Range | No. of analyses |
| Arginine | 6.3 | 6.5 | 6.4–6.6 | 2 | 6.1 | 7.0 | 5.7–10.1 | 9 |
| Histidine | 3.3 | 3.0 | 2.2–3.8 | 2 | 3.6 | 2.5 | 0.6– 4.4 | 9 |
| Isoleucine | 4.8 | 5.5 | 5.1–5.9 | 2 | 5.0 | 5.5 | 5.2– 5.9 | 4 |
| Leucine | 7.8 | 8.0 | 7.2–8.6 | 2 | 7.8 | 7.9 | 7.5– 8.6 | 4 |
| Lysine | 8.5 | 8.7 | 8.7–8.7 | 2 | 8.7 | 8.2 | 7.3–10.0 | 9 |
| Methionine | 2.5 | 2.9 | 2.4–3.4 | 3 | 2.7 | 3.3 | 2.5– 4.1 | 5 |
| Phenylalanine | 3.8 | 4.0 | 3.7–4.2 | 3 | 3.8 | 4.2 | 3.6– 4.9 | 6 |
| Threonine | 4.3 | 4.6 | 4.5–4.8 | 3 | 4.5 | 4.6 | 3.5– 5.3 | 5 |
| Tryptophan | 0.9 | 1.3 | 1.2–1.4 | 4 | 1.0 | 1.3 | 0.9– 1.5 | 11 |
| Valine | 5.2 | 5.4 | 5.3–5.4 | 2 | 5.2 | 5.2 | 5.1– 5.3 | 4 |

[1] This column was taken from table 3 and is, therefore, the average of all samples of each product.

[2] Literature values are from the paper of Lyman and Kuiken ('49).

The inter-laboratory agreement on results obtained from the 41 common samples assayed for 10 amino acids can be seen in table 2. To express the degree of variation among these results all values for a given amino acid were averaged, and the standard deviation between the two laboratories expressed as a percentage of this mean. The coefficients of variation so calculated ranged from 5.2% for histidine to 17.5% for tryptophan, with an average for all amino acids of 8.9%. It will be noted that different organisms were used

in the two laboratories to determine arginine, histidine, isoleucine, methionine, phenylalanine and threonine.

Similarly, the variation among replicate determinations made with a given test organism in this laboratory was found to range from 3.5% for leucine assayed with *L. delbrueckii* to 12.0% for methionine assayed with *L. fermenti,* with an average for all amino acids and all test organisms of 8.0%.

The results obtained in this laboratory when two organisms were used to determine the same amino acid in a series of samples were also compared. The variation between organisms was significantly greater than that between replicates obtained with the same organism in 4 out of the 10 cases for which data permitting such comparisons were available, namely, leucine, valine, and phenylalanine assayed with *L. arabinosus* and *L. delbrueckii,* and methionine assayed with *L. mesenteroides* and *S. faecalis.* However, the actual amino acid values obtained with different organisms showed a statistically significant difference in only two cases. The average phenylalanine content of 30 samples was found to be 3.45% (of the protein present) with *L. arabinosus* and 3.95% with *L. delbrueckii.* Similarly, the average valine content of 32 samples was 5.29% with *L. arabinosus* and 4.71% with *L. delbrueckii.*

It is felt that these comparisons provide strong evidence of the reliability of microbiological methods in determining the 10 amino acids studied.

### *The effect of heat processing*

The data for raw and heat-processed samples in table 2 show no significant effect of heat processing on the essential amino acid composition of the samples analyzed.


## SUMMARY

An inter-laboratory amino acid assay project has been described. A total of 61 raw and processed samples of fish and meat were assayed, of which 41 were analyzed at both the University of California at Los Angeles and the University of Wisconsin.

The essential amino acid composition of the various species of fish investigated was found to be in fair agreement with that found by earlier workers, although some differences are noted and discussed. Excellent agreement was found with respect to the 41 samples which were assayed in both laboratories. This comparison gives evidence of the reliability of the microbiological assay technique for amino acid analysis. Heat processing as employed in canning did not change materially the essential amino acid composition of the various fish and meat samples assayed.

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# THE INFLUENCE OF OPTICAL ISOMERISM AND ACETYLATION UPON THE AVAILABILITY OF TRYPTOPHAN FOR MAINTENANCE IN MAN [1]

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FIVE FIGURES

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Tryptophan is essential for the maintenance of nitrogen balance in man (Holt and others, '41; Rose, '47), hence must be present to make any protein or mixture of amino acids nutritionally complete. Since it is destroyed by the conditions usually employed in the acid hydrolysis of proteins, it must be added to acid hydrolysates designed for emergency use as protein substitutes. At present, the natural L form of tryptophan can be obtained commercially in limited quantities, but its cost is prohibitive in comparison with that of the synthetic DL modification. These circumstances obviously make it desirable to know how well the needs of the human subject for the L form can be met by supplying the DL mixture. It is with the comparative availabilities to man of the D and L isomers of tryptophan that this communication is chiefly concerned.

[1] The experimental data in this paper are taken from a dissertation submitted by Heber R. Baldwin in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa in February, 1949. These studies were made possible by a generous grant-in-aid from the Dow Chemical Company of Midland, Michigan, to whom we are also indebted for the supply of DL-tryptophan used.

Since this work was begun, about 30 months ago, Albanese, Davis and Lein ('48) have reported that D-tryptophan is unable to support nitrogen balance when it is fed to normal male infants as a component of DL-tryptophan, but that it becomes as effective as L-tryptophan when the racemic mixture is acetylated. This is quite the reverse of the situation which exists in the rat. In this species, D-tryptophan is utilized for growth almost as well as L-tryptophan; acetylation does not impair the availability of the L isomer but completely prevents the use of the D form for this purpose (du Vigneaud and others, '32; Berg, '34).

In support of their assertion that acetylation makes D-tryptophan available to the human infant for tissue synthesis, Albanese, Davis and Lein ('48) cite other data which seem too remotely related to be significant, involving as they do: (a) the excretion in the urine of an "aberrant" metabolite following the administration of 0.01 mol (about 2 gm) of DL-tryptophan to the normal human adult after an overnight fast (Albanese and Frankston, '44) and (b) failure to find the same unknown metabolite when acetyl-DL-tryptophan is fed, or to account for as much of the original 0.01 mol of acetylated derivative as when acetyl-L-tryptophan is given (Albanese, Frankston and Irby, '45; Albanese, Davis and Lein, '48). To infer that inability to account for a product fed indicates that it has therefore been converted into an available metabolite is quite unwarranted. In the instance cited, rupture of the indole ring would alone have sufficed to convert the acetyl-D-tryptophan into an available product; one which, moreover, might not be detectable by the method of assay employed. It is also important to note that mechanisms called into play to dispose of a relatively large excess of an amino acid supplied singly may differ markedly from those involved in the utilization of the relatively small quantity that need be supplied in the diet for maintenance and growth. This is certainly true of both free and acetylated D- and L-tryptophan in the rat, strongly probable in the rabbit and the

dog, and therefore a possibility that cannot safely be ignored in man.

The studies summarized in the present paper involve the responses of normal male adult subjects, as measured by nitrogen balance, to diets containing acid-hydrolyzed casein, with or without supplements of free or acetylated tryptophan, as the source of protein nitrogen. To make direct isomeric comparisons possible, free and acetylated D- and L-tryptophan

TABLE 1

*Physical properties and nitrogen content of the free and acetylated isomers of tryptophan*

| COMPOUND | m.p. (uncorr.)[1] | $[\alpha]_D^{20}$ [2] | | TOTAL NITROGEN [3] | |
|---|---|---|---|---|---|
| | | Found | Recorded (Berg, '33) | Found | Theory |
| | °C. | degrees | degrees | % | % |
| L-Tryptophan | 278–280 | — 32.0 | — 32.1 | 13.69 | 13.72 |
| D-Tryptophan | 278–280 | + 31.6 | + 32.45 | 13.66 | 13.72 |
| DL-Tryptophan | 279–282 | 0.0 | .... | 13.65 | 13.72 |
| Acetyl-L-tryptophan | 187 | + 26.0 | + 26.1 | 11.30 | 11.38 |
| Acetyl-D-tryptophan | 186 | — 25.2 | — 26.2 | 11.28 | 11.38 |

[1] The samples were heated in open capillary tubes. The temperature of the oil bath was raised 3°C. per minute.

[2] The aqueous solutions of tryptophan contained 0.5 gm per 100 ml of solution; the methanol solutions of acetyl-tryptophan, 1 gm per 100 ml of solution.

[3] Determined by the macro-Kjeldahl method.

were prepared by resolution for use along with the free DL mixture. So far as we are aware, data involving direct comparisons have not previously been published. A résumé of studies including similar tests has, however, very recently been presented by Rose ('49). With his findings our data are in essential agreement.

## EXPERIMENTAL

Data pertaining to the free and acetylated L- and D-tryptophan used in these tests are presented in table 1. These compounds were prepared from synthetic DL-tryptophan essentially as directed by Berg ('33).

*Tests with diets containing items low in nitrogen and tryptophan as sources of the non-protein requisites*

Serious efforts were made initially to devise a tryptophan-deficient diet in which an acid hydrolysate of casein [2] constituted the chief source of nitrogen, but the additional caloric requirements and the mineral and vitamin needs were to be met chiefly by commonly used dietary items, such as fruits, vegetables, fats, sugars and starches, with as little nitrogen or tryptophan as possible.[3] This approach had been used by Holt and his associates ('41) and was adopted in the hope that a palatable, somewhat varied, but fairly natural tryptophan-low diet could thus be provided. In the initial tests, removal of the daily supplement of tryptophan (250 mg) which had been fed during the 12 days allowed for adjustment to the new diet failed to produce a definitely negative nitrogen balance even after 12 days or more had elapsed. Therefore, to afford a more certain basis for selection, representative samples of the various items of the diet were homogenized, hydrolyzed with barium hydroxide and assayed for tryptophan by the microbiological method of Greene and Black ('44), in which lactic acid production by *Lactobacillus arabinosus* 17–5 is measured. The information thus acquired made it possible to detect and eliminate several unsuspected dietary items with a relatively high content of tryptophan. Assay of aliquots of the mixed diets indicated that they provided 135 mg of tryptophan per day. More rigid selection allowed a reduction in the tryptophan content to 115 mg per day, but the revised diet again failed to produce a distinctly negative balance when the initial supplements of tryptophan were removed. Since still further elimination of tryptophan

[2] We are grateful for the assistance of Dr. Charles F. Kade, Jr., and the Sterling-Winthrop Research Institute, Rensselaer, New York, and of Dr. John T. Correll and Dr. Curtis E. Meyer and the Upjohn Company, Kalamazoo, Michigan, who supplied us with sizable lots of acid-hydrolyzed casein.

[3] The advice of Dr. Kate Daum and Miss Evelyn Brandt of the Nutrition Department of the University Hospitals and their cooperation in making the facilities of the diet kitchen available to us in this phase of the study are gratefully acknowledged.

was impractical, this type of diet was abandoned in favor of one that was highly synthetic.

Although the preliminary studies outlined above failed to meet our needs, they did serve to emphasize that the minimum daily requirement of tryptophan is certainly low and that no appreciable deficiency is likely to be produced by the ordinary type of diet if enough is ingested to meet caloric needs.

### *Tests with synthetic diets*

The dietary regimen finally adopted was patterned after the type so successfully and extensively employed by Rose ('47). Table 2 provides information as to basic composition. In making the wafers, the dry ingredients were thoroughly mixed and the butter and commercial hydrogenated cottonseed oil [4] melted and stirred in. Approximately 4 l of water were then added, the batch was transferred to a Hobart mixer and stirred thoroughly for several minutes, and the batter was spread in small pools on cookie sheets and baked at 375°F. for 20 to 25 minutes.[5] The baked product was golden brown, almost paper-thin, crisp and tasty. For convenience, it was packaged in 100-gm lots and refrigerated until used. From the initial 7,670 gm of ingredients per batch, 6,500 to 6,800 gm of wafers were obtained. The amount of Cellu flour which they provided was usually sufficient to prevent the extremes of diarrhea and constipation. In a few instances, 5 to 10 gm more were supplied daily in water suspension.

Analyses of wafers from the various bakings showed that, per 100 gm, they contained approximately 52 mg of total N (by macro-Kjeldahl) and 1.5 mg of tryptophan (by the method of Greene and Black, '44). Values calculated from the analyses of the various individual items agreed well with those obtained by direct analyses of the mixtures. Components other than the acid-hydrolyzed casein and the supplements of free

[4] Crisco.

[5] We are greatly indebted to Dr. Sybil Woodruff and Miss McDivitt of the Home Economics Department for their counsel and the use of their equipment and kitchen facilities for mixing and baking these wafers.

TABLE 2

*Composition of the basal diet*

| DIETARY COMPONENT | DAILY ALLOTMENT | INGREDIENTS USED PER BATCH OF WAFERS | gm | VITAMINS PER CAPSULE | |
|---|---|---|---|---|---|
| Acid-hydrolysate of casein | 0.1 gm of N/kg of body wt. | Corn starch | 4,750 | A | 5,000 I.U. |
| | | Sucrose | 960 | D | 500 I.U. |
| | | Butter | 1,150 | Ascorbic acid | 37.5 mg |
| Wafers | 400–500 gm | Crisco | 60 | Thiamine HCl | 2.5 mg |
| Butter | 50 gm | Mineral mixture | | Riboflavin | 2.5 mg |
| Sucrose | 200 gm | (Murlin and others, '46) | 70 | Nicotinamide | 20.0 mg |
| Lemon juice | 100 ml | Sodium chloride | 120 | Pyridoxine HCl | 0.5 mg |
| Coffee | 300 ml | Baking powder [2] | 90 | Ca pantothenate | 5.0 mg |
| Vitamins [1] | | Cellu flour [3] | 470 | | |
| Upjohn Unicap | 1 capsule | | 7,670 | | |
| Folic acid | 5 mg | | | | |

[1] We are indebted to Dr. John T. Correll and the Upjohn Company, Kalamazoo, Michigan, for our supply of these vitamins.
[2] Composed of $NaHCO_3$, $Ca(H_2PO_4)_2 \cdot H_2O$, and corn starch in the ratio of 22:29:27 gm.
[3] Type B, Chicago Dietetic Supply House, Inc., Chicago, Illinois.

or acetylated tryptophan furnished less than 0.45 gm of total nitrogen per day, and together with the daily allotment of tryptophan-deficient hydrolysate, less than 25 mg of tryptophan.

Different lots of acid-hydrolyzed casein varied somewhat in taste and to a minor degree in nitrogen content, hence possibly also in amino acid composition. Each subject was therefore fed the same lot of hydrolysate throughout the experimental period. The daily allotment was weighed out, mixed with the tryptophan supplement when this was provided and dissolved in water. A third of this solution was consumed during each of the three meals. Enough wafers were eaten to raise the daily caloric intake to 42 Cal. or more per kilogram of body weight. In calculating caloric content, sucrose and starch were assumed to provide 4.0 Cal. each per gram, butter 8.0, the casein hydrolysate 4.0, and the wafers 4.3. Preliminary tests showed that in the subjects tested on this regimen, supplements of 200 mg of L-tryptophan per day were sufficient to establish nitrogen equilibrium.

Six normal male adults served as the experimental subjects in the final tests.[6] Of these, two were forced to discontinue the diet prematurely because of gastrointestinal distress characterized by nausea, anorexia, diarrhea and vomiting. The others had shown mild anorexia and nausea on the deficient diet, but not when tryptophan was provided.

Urine samples were collected from 8:00 A.M. to 8:00 A.M. on successive days. Each sample was analyzed for total nitrogen by the macro-Kjeldahl method. Creatinine N was determined routinely as a check on the collection of urines. Stools were collected daily and assayed for total nitrogen. To insure correspondence of fecal collections with the periods on the different diets, carmine was ingested as a marker each time the diet was changed. Average daily fecal nitrogen output per period was used in the calculations of nitrogen balance.

[6] To these and the several other experimental subjects who cooperated splendidly, we express sincere appreciation.

During the period of adjustment to the synthetic regimen, the basal diet was supplemented with 200 mg of L-tryptophan per day. From three to 14 days were required to attain nitrogen equilibrium, and 5 days more were usually allowed. Preliminary trials had shown that after this adjustment period responses to the omission or addition of tryptophan occurred promptly, hence that a period of 4 to 6 days was long enough to indicate whether a supplement could or could not be utilized to prevent or correct a negative nitrogen balance.

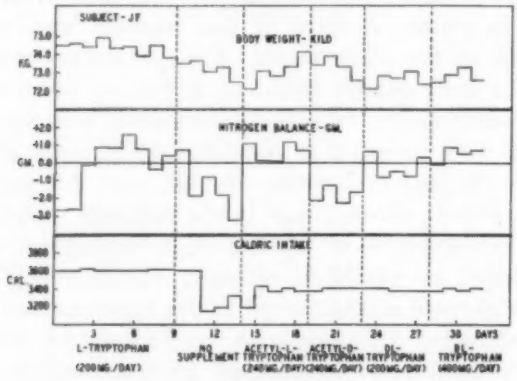


Figure 1

The data obtained on body weight, nitrogen balance and caloric intake in 5 of the 6 experimental subjects are compiled and presented in figures 1–5. Data on the 6th subject are omitted since he was forced to discontinue the diet during the initial period of adjustment.

## DISCUSSION OF RESULTS

Subject J F (fig. 1) maintained a fairly even caloric intake during his 32 days on experiment. Withdrawal of tryptophan produced a negative nitrogen balance. The accompanying drop in caloric intake to 43 Cal. per kilogram reflected the anorexia and nausea experienced during this period. The lowered caloric intake would probably have sufficed to main-

tain nitrogen equilibrium had the diet contained enough tryptophan. In this subject, 240 mg of acetyl-L-tryptophan (molecularly equivalent to 200 mg of free tryptophan) promoted a positive balance, but a similar amount of acetyl-D-tryptophan was without effect. A daily intake of 200 mg of DL-tryptophan failed to insure equilibrium. The response on 400 mg of DL-tryptophan was similar to that on 200 mg of the L-form or 240 mg of the acetyl-L-derivative. In general, the changes in body weight seemed to parallel approximately the changes in nitrogen balance.

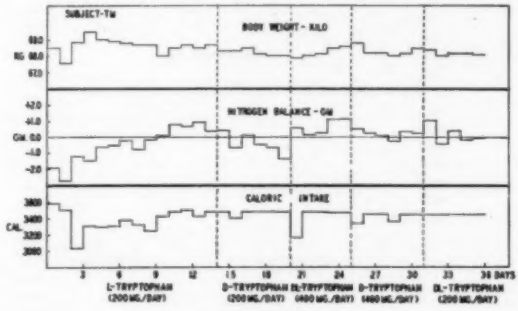


Figure 2

With few exceptions, subject T M (fig. 2) maintained an intake of 50 Cal. per kilogram per day throughout his 36 days on the study. As in the previous subject, 400 mg of DL-tryptophan were adequate for maintenance but 200 mg were not. The response to 200 mg of the D isomer was definitely negative, but to 400 mg it was only slightly inferior to that obtained on 200 mg of L-tryptophan. Changes in body weight were less marked in this subject, but again were roughly parallel to the changes in nitrogen balance.

The results obtained on W H (fig. 3) were not very satisfactory. On the 17th day this subject complained of gastrointestinal distress, nausea and anorexia. His stools were loose. The condition could not be cleared up, and the study was terminated on the 25th day by a siege of vomiting. This

subject's negative nitrogen balance during the period on D-tryptophan was undoubtedly attributable in part to the lowered caloric intake which began at the onset of his symptoms of distress.

The data obtained on subject L G (fig. 4) again indicate the inadequacy of a supplement of 200 mg of D-tryptophan per

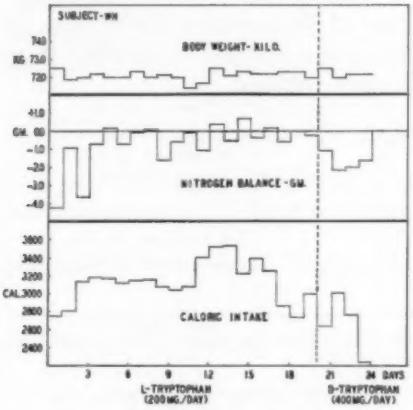


Figure 3

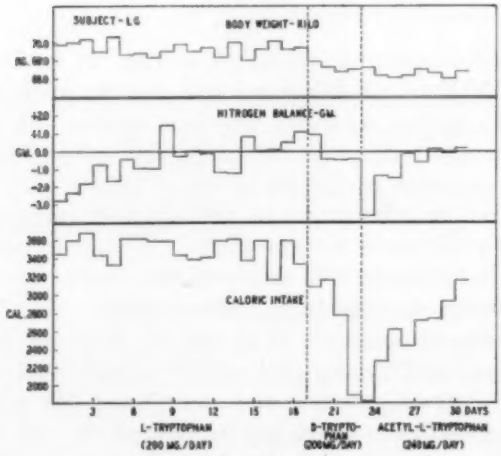


Figure 4

day. During the period on this supplement the subject experienced extreme lassitude, anorexia and nausea and could not be induced to maintain his previous caloric intake. Replacement of the D-tryptophan by an equimolar supplement of acetyl-L-tryptophan brought about a gradual return of appetite, an increased caloric intake and ultimately a restoration of nitrogen equilibrium.

The results obtained with subject R S (fig. 5) indicate again that acetyl-L-tryptophan is an effective substitute for

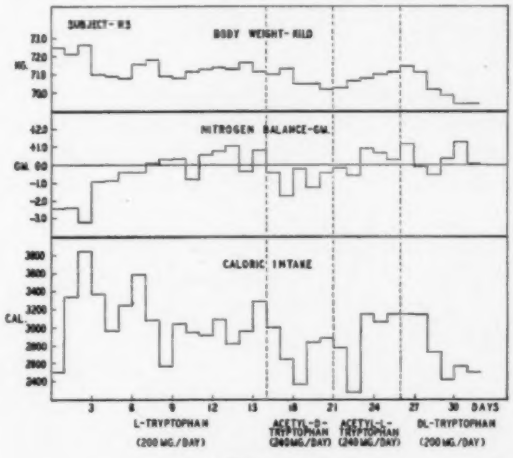


Figure 5

L-tryptophan and that 200 mg of DL-tryptophan are borderline or suboptimum in their effects. As in subject J F (fig. 1), the administration of 240 mg of acetyl-D-tryptophan per day led to a pronounced loss of appetite, an increasing nausea, and a negative nitrogen balance.

The subjective responses of the men to the various regimens were of interest, especially since none of them was informed of what to expect, either in advance of or during the change in type of diet or in level of supplementation. With a single exception (W H), none of the individuals was trained in biochemistry or nutrition or had any knowledge of symptoms

that had been encountered by others in similar series of tests. It is unlikely, therefore, that their reports of depressed appetite, nausea, increased nervous irritability, and so forth, were attributable to suggestion.

The difficulties encountered in attempting to maintain constant and adequate caloric intakes, especially during a period of dietary deficiency, are quite in line with observations made repeatedly on experimental animals. Similar symptoms have been reported by Rose ('47) in the human subject during periods of negative nitrogen balance.

At best, casein hydrolysates are far from tasty. Such untoward responses to their oral use as loss of appetite, nausea and decreased food intake have been noted (e.g., Co Tui, '46). Our 15 subjects, after the first few days, became accustomed, though perhaps never fully reconciled, to their strong taste. Several attempts were made to mask the objectionable flavor but these were ultimately abandoned in favor of the procedure of dissolving the hydrolysates in water, the subject drinking the solution and following this with a chaser of lemonade.

Holt and associates ('41) report that it sometimes required several days after the removal of the tryptophan supplement for subjects on their diets to go into negative nitrogen balance, also as long as a week after its replacement to restore nitrogen equilibrium. The rapid responses of our subjects to such changes can probably be attributed to a more rigorous exclusion of tryptophan from their basal diets and to their higher caloric intakes.

The studies reported in this paper were not designed to determine the amount of tryptophan needed to maintain nitrogen balance in the human adult, but the data recorded in figures 1 to 5, together with those from preliminary studies on 10 other subjects, indicate that nitrogen equilibrium can usually be maintained or restored on 225 mg of L-tryptophan per day and, in some instances, apparently on even less. In any event, the amount needed is certainly far less than the 6.0 to 9.0 mg per kilogram per day (420 to 630 mg for a 70-kg

subject) set by Holt and others ('44). Our data favor the minimum estimates of 250 mg to 150 mg per day suggested by Rose ('47, '49).

As indicated earlier, the deductions of Albanese, Davis and Lein ('48) concerning the availability of the D isomers in the male infant were based on responses to the free and acetylated DL mixtures. In general, our tests seem to indicate that DL-tryptophan is much less well utilized for maintenance than is L-tryptophan. The tests with D-tryptophan suggest that this component is certainly very poorly utilized, but leave some doubt that no use at all can be made of it, if the caloric intake is high and the amount ingested is sufficiently large. Our findings on acetyl-D-tryptophan do not agree with the conclusion of Albanese, Davis and Lein ('48) that the D-component of acetyl-DL-tryptophan can be utilized to maintain nitrogen balance. Their allowance of acetyl-DL-tryptophan was 48 mg per kilogram per day (259 to 356 mg per subject per day). The presence of the utilizable L isomer renders it more difficult to assess with certainty the effect of the D isomer alone. This would be particularly true if the amount fed appreciably exceeded the required minimum. At least in experimental animals, and presumably also in human subjects, borderline deficiencies are accompanied by variations in response which are wider and hence more difficult to evaluate than are the narrower differences noted when deficiencies are marked.

### *Excretion of tryptophan*

The apparent urinary excretion of free L-tryptophan, as measured by the Greene and Black ('44) microbiological method of analysis, without autoclaving with barium hydroxide, averaged 5.0 to 9.1 mg per day on the various supplemented regimens. After autoclaving the urine for 16 hours at 15 lb. pressure with 5 N barium hydroxide (and doubling the value obtained, to compensate for the fact that the test organism responds only to the L-component of the resulting racemic mixture), the average responses on the various regimens in-

dicated the following apparent outputs of free and combined tryptophan: on daily supplements of 200 mg of L-, DL- and D-tryptophan, respectively, 27.1, 38.5 and 72.6 mg per day; on daily supplements of 400 mg of DL- and D-tryptophan, 65.6 and 126.8 mg, respectively, per day; and on daily supplements of acetyl-L- and acetyl-D-tryptophan equivalent to 200 mg of tryptophan, 24.7 and 69.8 mg per day. To judge from the low values obtained with test solutions of acetyltryptophan, the last two figures may represent only half to three-fourths of the total excretion. It is therefore also possible that tryptophan bound in any other form difficult to hydrolyze may not have responded completely to the test; on the other hand, it is also possible that certain related intermediates or catabolites, or substances produced by the autoclaving, may respond and hence be assayed as tryptophan. Tests indicated that free D-tryptophan would beyond doubt have been completely racemized during the autoclaving with the barium hydroxide, hence would have been measured.

## SUMMARY AND CONCLUSIONS

Comparative tests have been made of the relative capacities of free and acetylated L- and D-tryptophan and of DL-tryptophan to support nitrogen equilibrium in the adult human subject when fed as supplements in a diet otherwise deficient in tryptophan.

Trials were first made of tryptophan-low basal diets containing a tryptophan-deficient hydrolysate of casein as the chief source of nitrogen, and also containing selected dietary items low in protein as the sources of additional calories and the non-protein requisites. This proved unsatisfactory because tryptophan is so widely distributed, even among dietary items relatively low in nitrogen, as to make it difficult to avoid its incidental inclusion in large enough amounts in this type of diet to prevent the production of a markedly negative nitrogen balance. Synthetic diets were therefore found preferable.

Diets providing 0.1 gm of nitrogen per kilogram per day in the form of an acid-hydrolysate of casein and enough more relatively pure carbohydrates and fats to raise the daily caloric intake to at least 42 Cal. per kilogram were subsequently employed. They contained approximately 25 mg of tryptophan and supported nitrogen equilibrium when supplemented with 200 mg of tryptophan per day. Nitrogen equilibrium was maintained in 15 subjects for periods of 12 days or more with total intakes of L-tryptophan not exceeding 225 mg each per day.

After the preliminary period of 8 to 19 days allowed for adjustment to the supplemented synthetic diet, responses to removal and replacement of the supplement were always fairly prompt, usually immediate, provided that a high caloric intake was maintained.

DL-tryptophan, in the amount of 400 mg per day, promoted responses equivalent to those noted on 200 mg of the L isomer, but the responses to 200 mg of DL-tryptophan per day were suboptimum. Daily supplementation with 200 mg of D-tryptophan failed to maintain nitrogen balance, but in one instance 400 mg per day seemed to promote a borderline response. There is little doubt that in the human subject the capacity of D-tryptophan to provide for maintenance is definitely inferior to that of L-tryptophan, but whether the D isomer could support nitrogen balance adequately, if fed in sufficiently large amounts in diets calorically adequate, is uncertain.

Acetyl-L-tryptophan in daily supplements (240 mg) equivalent to 200 mg of L-tryptophan appeared to be about as well utilized as the latter, but the response to 240 mg of acetyl-D-tryptophan was definitely negative. Acetylation does not enhance the availability of D-tryptophan for maintenance in the human adult.

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# THE MINIMUM RIBOFLAVIN REQUIREMENT OF THE INFANT [1]

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FOUR FIGURES

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The problem of ascertaining minimum dietary vitamin requirements has been complicated in recent years by the discovery of the antivitamins on the one hand and of the biosynthesis of vitamins by intestinal bacteria on the other. Vitamin requirements are also affected by disease. Hence, in considering requirements it is necessary to define the conditions under discussion. The present study was an attempt to throw light on the minimum riboflavin requirement of the healthy infant on a diet differing little from that customarily given to infants in this country.

## GENERAL PROCEDURE

The general plan adopted in this study was to feed infants a purified diet in which vitamins were supplied almost en-

[1] This study was supported in part by grants from the Sugar Research Foundation, the American Dairy Association, and the National Dairy Products Corporation.

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tirely in the form of accurately measured supplements. On such a diet the riboflavin content could be accurately controlled and altered at will. It was not deemed justifiable to reduce the riboflavin intake to a point at which symptoms of deficiency appeared. Instead, the attempt was made to determine the minimum riboflavin intake which would maintain the individual in health as judged by laboratory measurements of the riboflavin in blood and urine. Three measurements of blood riboflavin were made: free riboflavin plus flavin mononucleotide of the serum (called hereafter free riboflavin, since only traces of mononucleotide are present), total riboflavin of the white blood cells, and total riboflavin of the red blood cells. The total riboflavin of the serum, which includes the larger flavin adenine dinucleotide fraction, was also measured, but the values are not recorded since no consistent changes were observed throughout the experiment. This suggests that measurement of the total serum riboflavin would be of little help in detecting riboflavin deficiency.

The 24-hour urinary output of riboflavin was measured in order to ascertain the intake which would maintain but not exceed the minimum urinary excretion. The possible significance of this "point of minimum excretion" demands a word of explanation. Some of us (Najjar and Holt), when studying thiamine requirements in adults and adolescents, had found in 1943 that as the intake of this factor was reduced the urinary excretion fell sharply, until a certain critical minimum value was attained beyond which further reductions in intake produced only negligible further decreases in urinary output. This critical "point of minimum excretion" was found to occur at an intake two to three times greater than that which led to symptoms of deficiency. The point of "minimum urinary excretion" of thiamine has been determined for infants as well as older subjects, and has been used in estimating the thiamine requirement of the infant (Holt et al., '49). Further evidence indicating that this critical point corresponds closely with the minimum requirement for the maintenance of health was obtained from studies of tissue thiamine in rats at various levels of intake (Salcedo et al.,

'48). It was found that the brain is incapable of storing surplus thiamine when an excess of thiamine is fed. The thiamine content of the brain remains constant unless the quantity in the diet is reduced below the point of minimum urinary excretion, whereupon it falls sharply.

In the case of riboflavin data are not available correlating a point of critical urinary excretion to clinical manifestations of deficiency. Nevertheless it has been shown in older subjects (Najjar et al., '44) that a reduction in riboflavin intake reduced the urinary riboflavin to a minimum level, and this was not accompanied by clinical evidences of deficiency. The growing rat on a riboflavin deficient diet excretes an exceedingly small amount of riboflavin until sufficient riboflavin to permit maximum growth is provided, at which point the excretion is increased markedly (Burch et al., '48). It is recognized that methods for measuring urinary riboflavin are not entirely specific and that the very small "minimum" excretion on low riboflavin diets may consist in part—perhaps in large part—of non-riboflavin fluorescent substances. If this were the case, however, it would not vitiate the present argument. We therefore undertook to determine the point of minimum riboflavin excretion in the urine of the infant as a guide to the minimum requirement, since this would furnish a readily measurable end point presumably not far above the intake level leading to symptoms of deficiency.

## METHODS

### *Subjects*

Three male infants were used in this study. They were in good health except for mental deficiency. The mental state could be attributed in one to internal hydrocephalus, in the second to neonatal kernicterus; in the third it was without obvious explanation. The ages of these infants were 22, 14 and 32 months and they weighed 5.9, 8.6 and 9 kg, respectively, at the start of the experiment. They were placed on metabolism frames permitting separate collection of urine and feces for 5 days out of every 7 throughout the period of

study. Physical examinations with special reference to signs of riboflavin deficiency were carried out daily. Ophthalmologic examination, which included inspection of both the media and the fundus, and slit lamp examinations were repeated monthly throughout the experiment and sometimes more frequently. The subjects were weighed daily and measurements of length and head and chest circumferences were repeated at monthly intervals. X-rays of the long bones were taken at 4-week intervals and other clinical laboratory data, such as complete blood counts, urinalyses and electrocardiagrams, were obtained at frequent intervals.

### *Diet*

This consisted of vitamin-free casein,[5] commercial hydrogenated cottonseed oil,[6] and a vitamin-free dextrimaltose [7] in proportions to yield the following percentage distribution of calories: protein 15%, fat 35%, carbohydrate 50%. Each child received 100 cal. per kilogram of body weight daily. Minerals were supplied in the form of a mineral mixture.[8] Fat soluble vitamins were furnished by Oleum percomorphum,[9] 15 drops daily. The water soluble vitamins [10] were administered as a

[5] Labeo. This casein contains 0.35 μg of riboflavin per gram.

[6] Crisco. Contributed by Procter and Gamble.

[7] Specially prepared by Mead Johnson and Company through the courtesy of Dr. Warren M. Cox, Jr.

[8] The mineral mixture, supplied at the level of 5 gm per 750 cal. of diet, consisted of:

| | % | | % |
|---|---|---|---|
| NaCl | 18.9 | Fe citrate | 2.21 |
| $CaHPO_4$ | 25.0 | $CuSO_4$ (anhydrous) | 0.24 |
| $MgSO_4$ (anhydrous) | 6.86 | $MnSO_4$ (anhydrous) | 0.15 |
| $KHCO_3$ | 44.4 | KI | 0.015 |
| KCl | 2.88 | $Na_2F_2$ | 0.03 |

[9] Contributed by Mead Johnson and Company.

[10] The vitamin mixture, calculated in the case of the B factors to supply the same quantities as a quart of fresh cow's milk, gave the following daily quantities:

| | μg | | μg |
|---|---|---|---|
| Ascorbic acid | 25.0 | Inositol | 180.0 |
| Thiamine | 0.38 | Folic acid | .05 |
| Nicotinic acid | 0.87 | Para-aminobenzoic acid | 0.5 |
| Pantothenic acid | 3.5 | Choline | 147.0 |
| Pyridoxine | 0.67 | Riboflavin | Varied |

supplement which was divided into 4 equal portions given at 4 equally spaced intervals during the day.

### *Laboratory measurments*

Riboflavin in the urine was determined by the fluorometric method of Najjar ('41), using a Pfaltz and Bauer fluorophotometer. Total daily urines were collected in amber bottles and preserved with acetic acid. Measurements of the riboflavin in the stools were carried out from time to time to evaluate the factor of biosynthesis by the intestinal bacteria. The stools were preserved by adding 10 ml of glacial acetic acid to each 100 gm of stool. Extractions were performed according to the method of Najjar et al. ('44). Measurements of riboflavin in the red cells, white cells and serum were carried out by a microfluorometric procedure as previously described (Burch, Bessey and Lowry, '48). The bloods samples were drawn from the heel or finger tip.

### *Procedure*

It was felt, in all three of the cases, that time could be saved in approaching the "point of minimum urinary excretion" if riboflavin were completely omitted from the diet for a period instead of being gradually reduced. The complete omission was followed by a reduction in urinary output to a minimum level ranging between 33 and 50 μg per day. After these urinary figures had become stable, riboflavin was added to the diet in a stepwise manner until "spilling" in the urine occurred in quantities substantially above these figures. The intake was then alternately reduced and raised in order to determine the quantity ingested which would just sustain urinary excretion above the minimum level.

## RESULTS

The results are presented graphically in the accompanying charts (figs. 1, 2, 3 and 4) and tables. The protocols follow:

*Case I, J. G. (fig. 1)*

This study was a preliminary one which was incomplete and not carried out to its logical conclusion. It gave only an approximation of the range in which the end point lay. Although a significant excretion of riboflavin in the urine did not occur until the intake was increased to 0.6 mg daily, it seems quite likely that this excretion might have taken place if the 0.5 mg intake had been sustained for a longer period of time, since there was some increase in urinary riboflavin on the last day of this period.

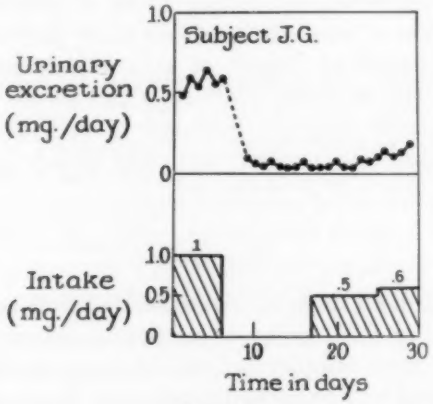



Figure 1

The data obtained thus indicated that the "point of minimum excretion" occurred at an intake of somewhat less than 0.6 mg daily; they were not sufficient to determine a more accurate end point.

At the time of this experiment, the methods for determining riboflavin in blood were not in operation and no data on blood levels were obtained.

*Case II, N. K. (fig. 2)*

After a period of 50 days in which riboflavin was entirely omitted and urinary excretion varied between 35 and 50 μg,

it was introduced again in increasing amounts until higher urinary figures were obtained. A gradual titration of the point of minimum urinary excretion was then carried out by adjusting the riboflavin intake until the minimum urinary excretion values were just exceeded. In this subject 0.40 mg of riboflavin daily gave the desired effect.

Blood determinations were not made at the onset of this study, the first being made on the 50th day of riboflavin withdrawal. Because of the delay in subsequently building up the patient to the point of "spilling," it is reasonable to conclude that the riboflavin-free period depleted the subject appreciably beyond the point of minimum excretion. It is noteworthy that at this time low values of free riboflavin in the serum and of total riboflavin in both the red and white cells were observed.

It is perhaps not possible to state with confidence the normal range for riboflavin in the elements of the blood. Burch, Bessey and Lowry ('48) reported for a dozen "well nourished" adults the following values: white cells, 252 μg% (range 227–293), red cells, 22 μg% (range 18–26), free riboflavin of the serum, 0.8 μg% (range 0.3–1.3). Data from infants receiving a normal infant diet showed values in the same range. The infant data are used as the basis for the "normal range" of figures 2 and 3. During the period of depletion the free riboflavin of the serum of infant N. K. was less than 0.1 μg%, and remained so for two weeks in spite of an intake of 0.4 to 0.8 mg riboflavin per day and in spite of a little "spillage" of riboflavin in the urine. However, with an average intake of about 0.35 mg per day the serum values gradually rose to 0.5 mg% after two months of realimentation. Similarly, the white cell and red cell values rose slowly. The white cell values appeared normal after two months, and the red cells after three. Thus, regardless of the significance of the concept of minimum point of excretion, this infant was apparently restored and maintained in riboflavin equilibrium with 0.4 mg riboflavin per day.

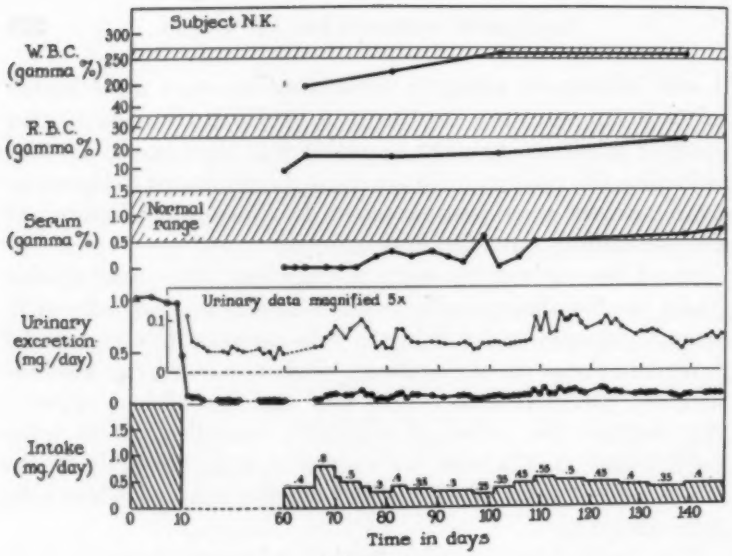
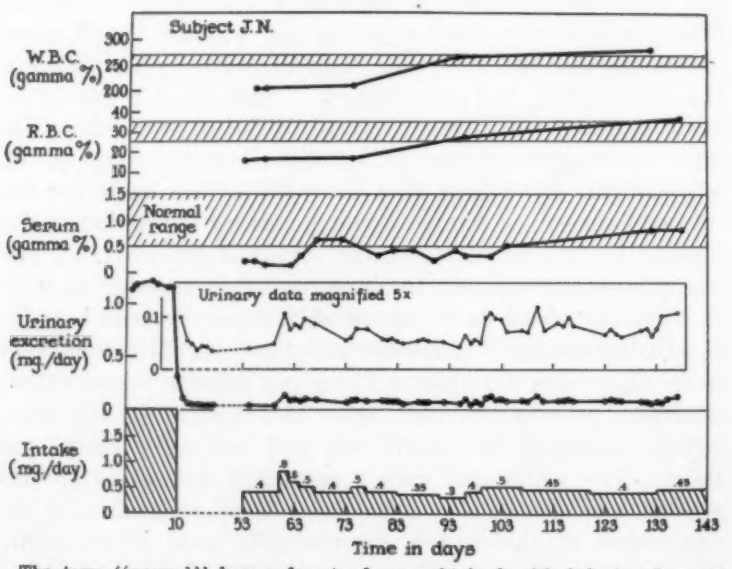



Figure 2




The term "normal" here refers to figures obtained with infants who were receiving a normal or conventional infant diet.

Figure 3

*Case III, J. N. (fig. 3)*

Riboflavin was withdrawn for 43 days and this resulted in urinary excretions varying between 33 and 40 μg daily. As previously described, "spilling" was obtained by increasing the riboflavin dosage and then carefully increasing and decreasing the amount ingested until the urinary output just exceeded the minimum figures. This point corresponded to an intake of 0.4 mg riboflavin daily in this subject also.

Blood studies were begun in this subject on the 43rd day without riboflavin. The initial values at the end of this period of depletion were comparable to those for the previous infant. The serum values for free riboflavin were a trifle higher but remained in general below 0.5 μg% until the white cells and red cells were restored to "normal," an interval of a month or two.

Figure 4 gives a composite picture of the average urinary riboflavin values occurring at the different levels of intake for all three subjects. Although the slope of the curve based on riboflavin excretion plotted against intake continues to increase up to an intake of 0.8 mg daily, it is clear that the beginning of the increased slope occurs at an intake of approximately 0.4 mg per day. We are inclined to interpret the higher excretion which begins at this level as evidence of a surplus of riboflavin beyond the requirements of the body.

*Riboflavin load tests.* These tests were performed at intervals on one of the above patients (subject J. G., weight 10 kg) using the procedure of Najjar and Holt ('41)—measuring the 4-hour excretion in the urine after the intravenous administration of 16 μg of riboflavin per kilogram. They illustrate a progressive retention of riboflavin after the "point of minimum excretion" is attained, and indicate that the load test has the advantage of revealing various degrees of deficiency beyond that point. Retentions in these studies varied from 99% to 68% of the 160 μg test dose. The greater retention occurred after depletion and was accompanied by a two-hour control excretion of 2.7 μg and a total 4-hour test

excretion of 7.8 μg. The smaller retention followed adequate intake of riboflavin; at this time the control value was 6 μg and the 4-hour figure was 63 μg.

Because of the possible contribution of intestinal bacteria to the riboflavin balance, the *riboflavin content of the stools* was studied routinely in our first subject and some isolated

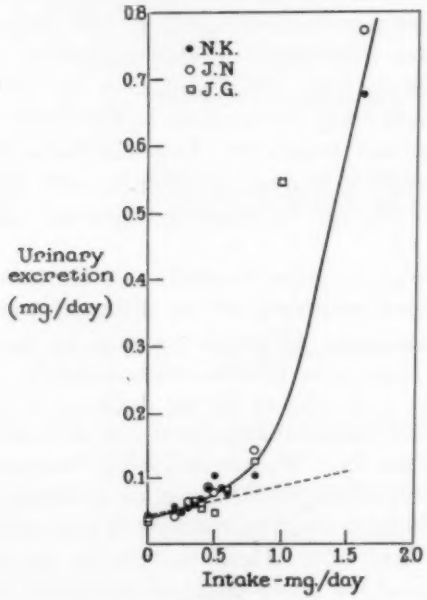



Figure 4

measurements were made in the other two infants. These observations are recorded in table 1. The total daily riboflavin content of the stool was found to vary widely in proportion with the stool weight, but the riboflavin concentration per gram of fresh stool remained singularly constant regardless of the reduction in intake to zero. It is thus obvious that biosynthesis of riboflavin occurs in the intestine of the infant, as in that of the adult. How much of this riboflavin is avail-

able for absorption cannot be ascertained from present data. To determine this point requires an effective method of inhibiting the growth of the organisms which synthesize riboflavin. The total stool excretion, however, is only a 6th of the amount that the present data suggest is necessary for maintenance.

The infants in these experiments made satisfactory gains in weight except toward the end of the study, when a flattening out of the weight curve occurred in each instance. The

TABLE 1

*Riboflavin content of the stool in relation to intake*

| SUBJECT | INTAKE | DAILY AMOUNT, AVER. | CONTENT OF STOOL | | |
|---|---|---|---|---|---|
| | | | Aver. at each intake level | Concentration | |
| | | | | Average | Aver. at each intake level |
| | *mg/day* | *μg* | *μg* | *μg* | *μg* |
| J.G. | 1.0 | 45.7 [1] | | 2 [1] | |
| | | 84.7 | | 4.5 | |
| | | 98.9 | | 2.3 | |
| | | 62.4 | 75.3 | 5.1 | 3.5 |
| | 0.6 | 40.3 | | 5.4 | |
| | 0.5 | 80 | | 4.2 | |
| | 0.4 | 86.5 | | 6.0 | |
| | | 76 | | 5.0 | |
| | | | | 4.0 | |
| | | 73.4 | 78.6 | 4.8 | 5.0 |
| | 0.3 | 66.3 | | 4.5 | |
| | | 68.9 | 67.2 | 4.7 | 4.6 |
| | 0.0 | 67.2 | | 5.0 | |
| | | 60.5 | | 5.0 | |
| | | 63.9 | | 4.2 | |
| | | 145.1 | 84.2 | 5.6 | 5.0 |
| N.K. | 0.0 | 120 | | 6.9 | |
| | | 34.7 | 77.3 | 3.9 | 5.4 |
| J.N. | 0.0 | 22.8 | | 2.5 | |

[1] Each of these figures represents the average of 5 individual daily figures.

thought that this failure to gain weight might be due to some degree of riboflavin deficiency, even though our chemical criteria in the blood and urine were satisfied, naturally occurred to us. Other evidence, however, has led us to dismiss this view and to attribute the phenomenon to some unknown deficiency in our purified diet. In the first place, we have noticed that all infants placed on our purified diet will thrive for only about 6 months, regardless of the vitamin mixture supplied; failure to gain weight will occur even on high riboflavin intakes, and in our subsequent experiments we now make a point of completing our observations early before this unknown deficiency manifests itself. Our observations on this unknown type of deficiency, which has been found to respond to brewers' yeast, are presented elsewhere. A second reason for exonerating riboflavin deficiency of responsibility for the development of stationary weight in the experiments reported here is the fact that in subsequent experiments carried out for another purpose we have been able to attain riboflavin equilibrium at the point of minimum excretion in a shorter period of time, and have been able to duplicate the urinary and blood findings here reported without encountering the arrest of weight.

## DISCUSSION

The data reported above indicate that in infants weighing 5.9 to 9 kg, fed as described, an intake of riboflavin of 0.4 to 0.5 mg per day is compatible with freedom from symptoms of riboflavin deficiency, maintenance of urinary riboflavin excretion at the critical point (the "point of minimum excretion"), and maintenance of presumed normal levels of riboflavin in the blood serum, red cells, and white cells. They do not permit one to state what the absolute minimum requirement is, even under these defined conditions.

The data suggest that the measurement of red cell riboflavin may be of service in assessing the riboflavin nutritional status. These values during depletion were lowered to less than half the probable normal value. The percentage change

was greater than for the white cells, which are also more difficult to measure. The serum values for free riboflavin might be useful, except that in the deficient zone the absolute values are exceedingly low and therefore difficult to measure with any accuracy.

We wish to emphasize the fact that we are not undertaking to recommend specific dietary allowances of riboflavin for infants on the basis of the data at hand. As we pointed out at the start, a requirement established by laboratory methods applies only to limited and well-defined conditions. On different diets and in various pathological states requirements may be quite different. Furthermore, any recommended allowance involves the further task of establishing a margin of safety, the proper size of which is an open question.

## SUMMARY

An intake of 0.4 mg of riboflavin per day by the infant served to maintain adequate blood levels, to maintain the urinary excretion at the upper limit of the zone of minimum excretion, and was not associated with clinical evidences of deficiency.

The limitations of these studies and the relation of the above figure to the minimum riboflavin requirements of the infant are discussed.

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# VITAMIN A UTILIZATION STUDIES

## I. THE UTILIZATION OF VITAMIN A ALCOHOL, VITAMIN A ACETATE AND VITAMIN A NATURAL ESTERS BY THE CHICK

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The present authors have undertaken a series of studies on vitamin A utilization with the particular purpose of elaborating the manner in which certain factors may affect differently the biological utilization of various esters and the free form of vitamin A.

Several investigators (Emmett and Bird, '37; Gray, Hickman and Brown, '40; Sobel et al., '48) have reported comparisons between the utilization of vitamin A esters and vitamin A alcohol under various experimental conditions. Their results indicate that the amount of the vitamin absorbed through the intestinal wall, and thereby made available to serve its essential physiological function in the metabolic processes of the animal, is dependent on a number of factors in addition to the quantity of vitamin A fed. These factors may be considered to fall into two groups, namely: (1) factors affecting the stability of the vitamin *in vivo* prior to absorption through the intestinal wall; and (2) agents or conditions which influence the intestinal absorption per se.

With respect to the factors affecting the stability of the vitamin, the interrelationship of vitamins A and E has been emphasized by Hickman et al. ('44), Gridgeman ('44) and Lemley et al. ('47). Their investigations are pertinent to the present study, however, only insofar as vitamin E may influence the utilization of vitamin A prior to its passage as the

free, unesterified form through the intestinal wall into the lymphatics and blood stream.

Studies on the mechanisms and mode of vitamin A absorption have been reported by Gray, Morgareidge and Cawley ('40), Clausen ('43) and Popper and Volk ('44). These investigations have shown that normally vitamin A esters are hydrolyzed prior to absorption but that, under certain physiological conditions which adversely affect the supply of the pancreatic enzymes to the duodenal contents, these esters are not hydrolyzed and absorption suffers.

It is therefore apparent that there exists a third group of factors, exerting favorable or adverse effects on the physiological mechanism of vitamin A ester hydrolysis, which would affect biological comparisons of dissimilar chemical forms of vitamin A.

We have emphasized in the present study the investigation of factors affecting the hydrolysis of vitamin A esters, particularly in respect to their varying effects on the utilization of differing forms of vitamin A as determined by liver storage measurements.

Chicks were used as the experimental species in the first part of our studies because of their relatively poor ability to utilize fats. We believed that for this reason factors affecting vitamin A ester hydrolysis could be more easily demonstrated with chicks than with other available experimental animals.

## METHODS

### *Preparation of vitamin A concentrate*

Vitamin A alcohol, acetate and natural ester concentrates were prepared in the laboratory, using a male soupfin shark liver oil as the source of vitamin A.

The vitamin A alcohol concentrate was obtained by saponifying the soupfin liver oil with alcoholic potassium hydroxide, extracting the unsaponifiable fraction with peroxide-free ethyl ether, and removing the solvent under reduced pressure. The vitamin A acetate was prepared by acetylating a portion of

the vitamin A alcohol. The natural ester concentrate was made by a process developed in our laboratories which does not involve the use of high vacuum distillation.

As the experiments described in this paper were conducted over an extended period of time, fresh preparations of the respective concentrates were made at various intervals during the course of the investigation. Since standardized procedures were used for their preparation, the various concentrates were extremely uniform in regard to spectrophotometric and chemical characteristics (table 1).

TABLE 1

*Physico-chemical characteristics of vitamin A concentrates fed*

| PHYSICO-CHEMICAL CHARACTERISTICS | SOUPFIN SHARK-LIVER OIL | VITAMIN A ALCOHOL | VITAMIN A ACETATE | VITAMIN A NATURAL ESTER |
|---|---|---|---|---|
| $E^{1\%}_{1\ cm}$ 328 mμ | 66.0 | 890–915 | 740–753 | 304–335 |
| Ratio $= \frac{E_{300\ m\mu}}{E_{328\ m\mu}}$ | 0.67 | 0.64–0.65 | 0.66–0.68 | 0.66–0.69 |
| Irrelevant absorption (%) | 10.0 | 6.0–8.0 | 6.0–9.0 | 8.0–11.0 |
| $L^{1\%}_{1\ cm}$ 620 mμ | 155.0 | 2140–2200 | 1600–1630 | 725–800 |
| Vitamin A alcohol (%) | | 99.0–100 | 2.0–4.0 | 3.0–6.0 |
| Vitamin A acetate (%) | | | 96.0–98.0 | |
| Vitamin A natural ester (%) | 100.0 | 0.0–1.0 | | 94.0–97.0 |

The ultraviolet spectrophotometric measurements were made on a Beckman spectrophotometer, using absolute ethanol as a solvent. The proportion of irrelevant absorption present in the spectrophotometric curves in the region of the vitamin A absorption maximum was calculated according to the method devised by Morton and Stubbs ('46), using the fixation wave lengths specified for the solvent ethanol. The $L^{1\%}_{1\ cm}$ 620 mμ values were measured on an Evelyn photoelectric colorimeter according to the procedure of Dann and Evelyn ('38). Percentages of the alcohol and ester forms of vitamin A in the respective concentrates were determined by the procedures

described by Reed et al. ('44). The concentrates were stored under nitrogen and refrigeration.

*Preparation of vitamin A dilutions for feeding*

Vegetable oils and other materials used as diluents were high quality commercial and pharmaceutical products. The vegetable oils contained a minimum of peroxides and showed no evidences of rancidity.

The dilutions of the vitamin A concentrates for feeding were calculated on the basis of spectrophotometric units of vitamin A ($E^{1\%}_{1\ cm}$ 328 mμ × 2,000). The dilutions were assayed before and after the test feeding period to determine if any change in potency had taken place.

*Basal diet*

White Leghorn and New Hampshire chicks were used according to their availability for the experiments. The one-day old chicks were placed on a low vitamin A diet (Almquist et al., '43), with several modifications. The composition of the basal ration is shown in table 2.

The incorporation of the indicated amount of vitamin A into the diet was sufficient to maintain the chicks at a level at which no visible symptoms of vitamin A deficiency were apparent, and no significant storage of the vitamin A occurred in the liver.

The chicks were housed in electrically heated battery brooders under uniform conditions of light, temperature, and so forth. The basal feed and water were supplied ad libitum. At 50 to 56 days of age the chicks were divided into comparable groups according to sex and weight and placed on test.

*Vitamin A liver storage measurements*

The livers from each group of chicks were weighed individually, pooled and homogenized in a Waring Blendor, and samples were taken for the determination of vitamin A. A

modified procedure based on the method described by Glover et al. ('47) was used for all liver assays.

A 5- to 10-gm sample of liver tissue was thoroughly mixed with approximately 4 times its weight of anhydrous sodium sulfate. The mixture was extracted with 4 portions, of 50 ml each, of redistilled petroleum ether (B.P. 40 to 60°C.). The extract was filtered into a 250-ml volumetric flask, the filter paper being washed with small portions of petroleum ether

TABLE 2

*Composition of the basal ration*

| BASAL MIXTURE | | VITAMIN SUPPLEMENTS | |
|---|---|---|---|
| | % | | *per lb. basal mixture* |
| Fish meal (extracted sardine meal) | 15.00 | Vitamin D (Delsterol) | 454 A.O.A.C. units |
| Brewers' yeast | 7.50 | Vitamin K (2-methyl naphthoquinone) | 0.18 mg |
| Wheat bran | 10.00 | Vitamin E (21.3% mixed tocopherols) | 11.0 mg |
| Oyster shell | 0.50 | Vitamin A (shark liver oil) | 1,200 spec. units |
| Salt | 1.00 | | |
| Cottonseed oil (Wesson) | 2.00 | | |
| Ox bile salts | 0.20 | | |
| Rice flour (polished rice) | 63.54 | | |
| Choline chloride | 0.20 | | |
| Manganese sulfate (85%) | 0.06 | | |
| | 100.00 | | |

to remove traces of vitamin A. The volume was adjusted to 250 ml and a suitable aliquot taken and evaporated to dryness by a stream of nitrogen. The residue was dissolved immediately in 1 ml of chloroform and the vitamin A estimated by the Carr-Price reaction, using an Evelyn colorimeter equipped with a 620 mμ filter. The colorimeter was calibrated against the U.S.P. Vitamin A Reference Standard for determining the vitamin A values reported. This calibration was found to be the same as when the instrument was calibrated using the

soupfin shark liver oil described above at an assumed potency of $E\,{}^{1\%}_{1\,cm}$ 328 mμ × 2,000.

We found that the livers of the chicks contained insignificant amounts of the carotinoid pigments, and therefore no corrections for the presence of these compounds were necessary in computing the vitamin A content of the livers.

The vitamin A values reported in this investigation are the result of duplicate determinations which agreed within the limits of ± 3%.

The liver samples were stored under refrigeration until assayed for vitamin A. Control analyses made of various samples indicated no significant destruction of vitamin A during the period of storage.

### *Experiment 1*

This experiment was originally part of a series designed to observe the effects of certain anti-oxidants on the relative utilization of the alcohol, acetate and natural ester forms of vitamin A. In addition, the effect was studied of administering various quantities of an oxidized diluent oil together with the respective vitamin A preparations. A full discussion of these specific considerations is beyond the scope of the present paper and will be presented elsewhere. However, since the data derived from this particular experiment provide an indication of the inhibiting action of relatively large amounts of diluent fat on the utilization of vitamin A esters, the experiment is included in this report.

White Leghorn chicks, 50 days of age, were assigned to experimental groups of 9 each. The dilutions of the respective vitamin A concentrates in cottonseed oil and oxidized corn oil were administered on three successive days, 0.1 ml or 2.0 ml of the respective supplement being fed each day orally by means of a calibrated tuberculin syringe. Each chick received a total of 27,600 units of vitamin A for the three-day period. On the 4th day, or 72 hours after receiving the first supplement, the chicks were killed and the livers removed. The livers of each

group were combined and the vitamin A content determined on the composite sample. The vitamin A liver storage measurements are listed in table 3.

The oxidized corn oil used as a diluent was prepared by aerating the oil for 24 hours at room temperature. Fresh dilutions of the vitamin A preparations were made daily in order to prevent destruction of vitamin A by oxidation.

### *Experiment 2*

New Hampshire chicks, 52 days of age, were divided into comparable groups of 10 chicks each. Dilutions of the alcohol,

TABLE 3

*Experiment 1 — Vitamin A liver storage by the chick* [1]

| DILUENT | COTTONSEED OIL NO ANTI-OXIDANTS | | | COTTONSEED OIL WITH ANTI-OXIDANTS [2] | | | AERATED CORN OIL NO ANTI-OXIDANTS | | | AERATED CORN OIL NO ANTI-OXIDANTS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Volume and potency of suppl. fed daily | 0.1 ml @ 92,000 units/ml | | | 0.1 ml @ 92,000 units/ml | | | 0.1 ml @ 92,000 units/ml | | | 2.0 ml @ 4,600 units/ml | | |
| Total units fed in 3-day period | 27,600 | | | 27,600 | | | 27,600 | | | 27,600 | | |
| Group no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Form of vitamin A | Alcohol | Acetate | Ester | Alcohol | Acetate | Ester | Alcohol | Acetate | Ester | Alcohol | Acetate | Ester |
| Average wt. of livers (gm) | 16.9 | 16.4 | 17.5 | 15.8 | 15.1 | 18.6 | 16.5 | 16.8 | 15.7 | 16.2 | 18.3 | 14.6 |
| Units of vitamin A per liver | 6,352 | 9,422 | 8,680 | 6,178 | 9,362 | 8,128 | 6,600 | 7,963 | 7,018 | 7,776 | 7,851 | 6,220 |
| Vitamin A stored (%) | 23.0 | 34.1 | 31.4 | 22.4 | 33.9 | 29.4 | 23.9 | 28.9 | 25.4 | 28.2 | 28.4 | 22.5 |

[1] Control groups, receiving diluent oils only as a supplement, showed no liver storage of vitamin A.

[2] Nordihydroguaiaretic acid 0.01% and citric acid 0.05%.

TABLE 4

*Experiment 2 — Liver storage in chicks fed daily supplement of 10,000 units vitamin A in various diluent oils* [1]

| DILUENT | COTTONSEED OIL | | | CORN OIL | | | SARDINE OIL | | | BASKING SHARK OIL | | | MINERAL OIL U.S.P. | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Vol. and potency of suppl. fed daily | 2.0 ml @ 5,000 units/ml | | | 2.0 ml @ 5,000 units/ml | | | 2.0 ml @ 5,000 units/ml | | | 2.0 ml @ 5,000 units/ml | | | 2.0 ml @ 5,000 units/ml | | |
| Total units fed in 3-day period | 30,000 | | | 30,000 | | | 30,000 | | | 30,000 | | | 30,000 | | |
| Group no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| Form of vitamin A | Alcohol | Acetate | Ester | Alcohol | Acetate | Ester | Alcohol | Acetate | Ester | Alcohol | Acetate | Ester | Alcohol | Acetate | Ester |
| Average wt. of livers (gm) | 17.8 | 18.0 | 17.4 | 17.8 | 16.8 | 18.6 | 17.4 | 18.3 | 16.7 | 18.8 | 18.0 | 18.6 | 19.6 | 17.9 | 17.0 |
| Units of vitamin A per liver | 7,490 | 6,830 | 5,320 | 7,150 | 5,970 | 4,940 | 3,930 | 3,960 | 1,390 | 4,510 | 2,390 | 1,880 | 4,860 | 2,160 | 1,465 |
| Vitamin A stored (%) | 25.0 | 22.8 | 17.7 | 23.8 | 19.9 | 16.5 | 13.1 | 13.2 | 4.6 | 15.0 | 8.0 | 6.3 | 16.2 | 7.2 | 4.9 |

[1] Control groups, receiving diluent oils only as a supplement, showed no liver storage of vitamin A.

acetate and natural ester concentrates containing 5,000 vitamin A units per milliliter were made in cottonseed oil, corn oil, sardine oil, basking shark oil and mineral oil. The supplements contained 0.01% by weight of hydroquinone as an anti-oxidant.

Each chick received orally 2.0 ml of a vitamin A supplement daily for three successive days, or a total of 30,000 units of the vitamin. The chicks were sacrificed on the 4th day and the livers composited and assayed for vitamin A as described for the preceding experiment. The results are shown in table 4.

### *Experiment 3*

White Leghorn cockerels, 56 days of age, were assigned to groups of 12 chicks each. Dilutions of the respective vitamin A forms containing the specified units of vitamin A per milliliter were prepared in corn oil, castor oil, jojoba seed oil and ethyl laurate. The composition and chemical characteristics of jojoba seed oil are described by Jamieson ('43). The supplements contained 0.5% by weight of mixed tocopherols as an anti-oxidant.

The chicks received their daily supplements of the vitamin A-containing preparations as described for the previous experiments. A total of 30,000 vitamin A units was administered to each chick for the three-day test period.

The livers from each group of chicks were divided into 4 subgroups containing three livers each and assayed for vitamin A. The results are listed in table 5.

The data derived from this experiment were analyzed statistically in order to determine the mathematical significance of the values obtained.

## DISCUSSION OF RESULTS

The experimental results, which are discussed in detail in the following paragraphs, clearly demonstrate the existence of factors affecting the hydrolysis of the ester form of vitamin A at high levels of intake under various test conditions. The

TABLE 5

*Experiment 3 — Relationship between chemical forms of vitamin A and diluent oils, as measured by liver storage by the chick* [1]

| DILUENT | CORN OIL | | | CORN OIL | | | CASTOR OIL | | |
|---|---|---|---|---|---|---|---|---|---|
| Volume and potency of suppl. fed daily | 2.0 ml @ 5000 units/ml | | | 0.1 ml @ 100,000 units/ml | | | 2.0 ml @ 5000 units/ml | | |
| Total units fed in 3-day period | 30,000 | | | 30,000 | | | 30,000 | | |
| Group no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Form of vitamin A fed | Alcohol | Acetate | Ester | Alcohol | Acetate | Ester | Alcohol | Acetate | Ester |
| Sub-group i (units) | 10,105 | 9,718 | 6,507 | 10,293 | 10,604 | 8,275 | 7,269 | 7,444 | 4,560 |
| Sub-group ii (units) | 12,657 | 10.743 | 8,720 | 11,390 | 11,751 | 7,878 | 7,592 | 8,852 | 4,117 |
| Sub-group iii (units) | 11,474 | 12,366 | 7,162 | 11,224 | 11,598 | 8,024 | 9,002 | 8,212 | 5,684 |
| Sub-group iv (units) | 8,159 | 7,482 | 6,134 | 8,614 | 12,804 | 9,109 | 8,972 | 7,582 | 5,994 |
| Avg. vit. A per liver | 10,667 | 10,077 | 7,131 | 10,380 | 11,689 | 8,322 | 8,209 | 8,023 | 5,089 |
| Avg. vit. A stored (%) | 35.6 | 33.6 | 23.8 | 34.6 | 39.0 | 27.7 | 27.4 | 26.7 | 17.0 |
| DILUENT | JOJOBA SEED OIL | | | JOJOBA SEED OIL | | | ETHYL LAURATE | | |
| Volume and potency of suppl. fed daily | 2.0 ml @ 5000 units/ml | | | 0.1 ml @ 100,000 units/ml | | | 2.0 ml @ 5000 units/ml | | |
| Total units fed in 3-day period | 30,000 | | | 30,000 | | | 30,000 | | |
| Group no. | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
| Form of vitamin A fed | Alcohol | Acetate | Ester | Alcohol | Acetate | Ester | Alcohol | Acetate | Ester |
| Sub-group i (units) | 8,540 | 4,584 | 3,133 | 9,379 | 10,321 | 7,112 | 6,969 | 4,462 | 3,400 |
| Sub-group ii (units) | 9,694 | 4,354 | 2,028 | 8,834 | 10,606 | 7,708 | 9,550 | 4,518 | 4,266 |
| Sub-group iii (units) | 10,291 | 4,972 | 2,341 | 8,400 | 11,043 | 10,205 | 9,143 | 4,807 | 4,217 |
| Sub-group iv (units) | 10,876 | 5,157 | 2,255 | 10,052 | 9,990 | 8,565 | 9,648 | 3,170 | 2,744 |
| Avg. vit. A per liver | 9,850 | 4,767 | 2,439 | 9,166 | 10,490 | 8,398 | 8,828 | 4,239 | 3,657 |
| Avg. vit. A stored (%) | 32.8 | 15.9 | 8.1 | 30.5 | 35.0 | 28.0 | 29.4 | 14.1 | 12.2 |

[1] A difference of 1,507 is required for significance (5% level) between any two of the following test groups: 1, 2, 3, 7, 8, 9, 10, 11, 12, 16, 17 and 18. A difference of 1,943 is required for significance (5% level) between any two of the following test groups: 1, 2, 3, 4, 5, 6, 10, 11, 12, 13, 14 and 15.
Control groups, receiving diluent oils only as a supplement, showed no liver storage of vitamin A.

importance of these factors at lower levels of vitamin A intake was also demonstrated in preliminary and additional experiments performed in this laboratory. The earlier tests were designed primarily to determine the vitamin A dosage required for optimum liver storage of the vitamin. In feeding 2,500 units daily, as compared to the 10,000 unit level fed in the experiments described in this paper, the differences in availability of the various forms of vitamin A due to hydrolytic factors were of the same order. However, further investigations are necessary to establish definitely the quantitative effects of these factors at the vitamin A levels of intake recommended for normal nutrition.

*Experiment 1*

Although the significance of the results shown in table 3 cannot be demonstrated by statistical treatment, the data are remarkably uniform and show the same relative utilization of the three forms of vitamin A in all the groups fed a 0.1 ml supplement. The data indicate that factors which might be expected to influence stability *in vivo,* i.e., anti-oxidants and pro-oxidants, actually had little or no effect. The slightly lesser utilization of the natural ester and acetate forms in the aerated corn oil as compared to the cottonseed oil dilutions seems to be due to normal characteristics of the corn oil (see also experiment 2) rather than to the presence of peroxides. This observation with respect to the relatively small importance of factors affecting stability *in vivo* in vitamin A utilization under the conditions of these experiments is also confirmed by a number of other exploratory experiments performed in this laboratory.

The second point of interest is the reversal of the relative utilization of the alcohol and natural ester forms as between the 0.1 ml and the 2.0 ml dilutions in corn oil.

*Experiment 2*

Foy and Morgareidge ('47), in their investigations concerning the Guggenheim and Koch ('44) method of bioassay for

1, 2, 3, 4, 5, 6, 10, 11, 12, 13, 14 and 15: Control groups, receiving diluent oils only as a supplement, showed no liver storage of vitamin A.

vitamin A, have demonstrated that variations in vitamin A storage in the livers of rats can be produced by varying the diluent in which the vitamin A dosage is fed. Our experiments confirm that this observation is also true for chicks.

A statistical analysis of the data in table 4 shows that the direct effects of the form of vitamin A, and of the vegetable oils versus the other carriers, exceeded the interaction between these two factors very significantly.

It is to be noted that the utilization of each of the three vitamin forms is only slightly affected by the use of corn oil as a diluent instead of cottonseed oil.

TABLE 6

*Experiment 2 — Analysis of variance of vitamin A liver storage data listed in table 4*

| SOURCE OF VARIATIONS | DEGREES OF FREEDOM | MEAN SQUARE | VARIANCE RATIO |
|---|---|---|---|
| Form of vitamin A | 2 | 8,380,305 | 23.64 |
| Vegetable oils vs. others | 1 | 39,983,335 | 112.79 |
| Other comparisons among carriers | 3 | 184,579 | 0.52 |
| Form of vitamin A × carrier | 8 | 354,501 | 1.00 |

On the other hand, the use of sardine body oil, basking shark liver oil or mineral oil has a marked effect on the utilization of all three forms with respect to their utilization from cottonseed oil diluent. The adverse effect of these carriers is more marked in the cases of the vitamin A acetate and vitamin A natural ester than it is in the case of the vitamin A alcohol, except for vitamin A acetate in sardine oil.

Sardine body oil, basking shark oil and mineral oil were chosen because we believed they might have an adverse effect on the hydrolysis of vitamin A esters. It is apparent that they do, although they also inhibit absorption per se. We believe that the mechanism of interference with vitamin A ester hydrolysis is different in the case of sardine oil than in those of basking shark liver oil and mineral oil, being connected in the first case with the presence of glycerides of unsaturated

fatty acids and in the second with the presence of large quantities of hydrocarbons.

### *Experiment 3*

The data in table 5 were analyzed statistically in two separate treatments. An analysis of variance was made of the results for groups 1 through 3, 7 through 12, and 16 through 18. A separate analysis of variance was made of the results for groups 1 through 6 and 10 through 15. These treatments are illustrated in table 7.

TABLE 7

*Experiment 3 — Analyses of variance of vitamin A liver storage data shown in table 5*

| SOURCE OF VARIATIONS | DEGREES OF FREEDOM | MEAN SQUARE | VARIANCE RATIO |
|---|---|---|---|
| (1) Groups 1, 2, 3, 7, 8, 9, 10, 11, 12, 16, 17 and 18 | | | |
| Carrier (diluent) | 3 | 35,976,381 | 56.67 |
| Form of vitamin A | 2 | 92,754,103 | 146.10 |
| Sub-groups | 3 | 3,557,119 | 5.60 |
| Carrier × form of vitamin A | 6 | 7,797,712 | 12.28 |
| Pooled error | 33 | 1,097,958 | 1.00 |
| (2) Groups 1, 2, 3, 4, 5, 6, 10, 11, 12, 13, 14 and 15 | | | |
| Carrier (diluent) | 1 | 57,693,445 | 31.21 |
| Form of vitamin A | 2 | 52,368,750 | 28.33 |
| Volume of supplement | 1 | 60,871,561 | 32.93 |
| Carrier × volume | 1 | 23,978,614 | 12.97 |
| Form of vitamin A × volume | 2 | 22,492,444 | 12.17 |
| Pooled error | 40 | 1,848,383 | 1.00 |

Comparisons may be made of the utilization in the various carriers of all forms of vitamin A taken together. Comparisons may also be made of the relative utilization of the three forms of vitamin A, taking all types of carrier together. Such comparisons are, however, of minor practical value. Comparison between various pairs of groups is the most valuable for the purpose of drawing inferences.

In the case of groups 1 through 3, 7 through 12, and 16 through 18, it is possible to make 66 comparisons of two groups taken at a time. Forty-nine of these disclose statistically significant differences; 17 do not. Of the 49, 20 comparisons are believed by us to be useful, namely: group 1 with 3, 7 and 16; 2 with 3, 8, 11 and 17; 3 with 9, 12 and 18; 7 with 9 and 10; 8 with 11; 9 with 12; 10 with 11 and 12; 11 with 12; and 16 with 17 and 18. Of the 17 comparisons which do not disclose a statistically significant difference, we believe the following to be useful: 1 with 2 and 10, 7 with 8 and 16, 9 with 18, 10 with 16, 11 with 17, 12 with 18 and 17 with 18.

A typical and valuable line of reasoning which can be applied to these comparisons follows:

1. In the comparisons of group 1 with 3, 7 with 9, 10 with 12 and 16 with 18, the utilization of vitamin A alcohol is compared to the utilization of vitamin A natural esters in the identical carrier. As vitamin A esters are hydrolyzed prior to absorption, the effect of a particular carrier on absorption per se would be the same with respect to vitamin A alcohol as with respect to vitamin A esters. As the esters have been shown by other investigators to be more stable *in vivo* than vitamin A alcohol, their significantly lower utilization in each of the mentioned comparisons can be due only to factors having to do with hydrolysis.

2. The comparison of group 1 with group 10 shows that jojoba seed oil does not have a significant depressive effect on the utilization of vitamin A alcohol as compared with corn oil. As hydrolysis is not involved here, it is shown that the difference between the combined effect of the stability and absorption per se factors in jojoba oil versus that of the same factors in corn oil is not significant.

3. In the comparisons of group 2 with group 11 and group 3 with group 12, absorption per se factors would have the same effect as in the comparison of groups 1 and 10, while stability factors would have even less effect. Therefore, the very significant lowering in the utilization of acetate and natural ester forms in the jojoba oil versus the corn oil dilutions is

shown to be due to the presence of more anti-vitamin A hydrolysis factors (qualitatively or quantitatively) in the jojoba oil.

Following similar lines of reasoning, it can be shown that the inferior utilization of all forms of vitamin A from ethyl laurate versus corn oil demonstrates: (a) greater inhibition of hydrolysis by the ethyl laurate than by corn oil; and (b) the former's adverse effect on stability *in vivo* or absorption per se or both.

Castor oil under the conditions of these experiments is shown to be inferior to corn oil as a diluent in vitamin A feeding, due to factors affecting stability *in vivo* or absorption per se, or both, with the latter factor almost certainly the more important.

In case of groups 1 through 6, and 10 through 15, it is possible to make 66 comparisons of two groups taken at a time. Thirty-seven of these comparisons disclose statistically significant differences. Of these 37, the most important (eliminating duplicate comparisons from the first group-set) are groups 4 with 6, 5 with 6, 12 with 15 and 14 with 15. Of the 29 comparisons which do not disclose statistically significant differences, the following are also of special interest: group 1 with 4, 2 with 5, 3 with 6, 4 with 5 and 13, 5 with 14, 10 with 13, and 13 with 14 and 15.

It happens that every comparison duplicated in the two group-sets, if significantly different according to the first criterion (1,507 units), is also statistically significant according to the second criterion (1,943 units).

A reduction in the volume of the jojoba seed oil from 2.0 ml to 0.1 ml significantly increases the utilization of vitamin A acetate and vitamin A natural ester. As the same change in volume of jojoba seed oil has no significant effect on the utilization of vitamin A alcohol, it can be seen that the improved utilization of the ester forms in the 0.1 ml dilutions is due to the lesser amounts of hydrolysis-inhibiting factors carried in this quantity of diluent.

Although the differences between the 2.0 ml and 0.1 ml corn oil dilutions with respect to utilization of the various forms of the vitamins are not statistically significant, there is indication that the reduction of corn oil volume similarly tends to reduce the already small amount of hydrolysis-inhibiting factors present.

The comparisons of group 1 with group 4 and groups 10 with 13 show that factors affecting absorption per se and stability are of minor importance in both corn oil and jojoba oil, unless these factors operate in opposing directions with substantially equal magnitude.

## SUMMARY

1. Vitamin A liver storage tests were used as the criterion for determining the relative utilization of the alcohol, acetate and natural ester forms of vitamin A by the chick under various experimental conditions.

2. Wide variations in the storage of vitamin A in the livers of chicks were found, depending both on the form of vitamin A fed and on the character and quantity of diluent. The experimental data were subjected to analyses of variance, and the statistical significance of a great part of the data was established. The importance was demonstrated of factors affecting the hydrolysis of vitamin A esters in vitamin A utilization. The following carriers were shown to contain factors inhibiting vitamin A ester hydrolysis: jojoba seed oil, ethyl laurate, basking shark liver oil and mineral oil. Sardine oil was shown to contain factors which interfere with the hydrolysis of vitamin A natural esters, although not with the hydrolysis of vitamin A acetate. Although statistical significance was not demonstrated, it appears likely that cottonseed oil and corn oil also contain small amounts of factors which interfere with vitamin A ester hydrolysis.

The existence of factors affecting vitamin A absorption per se was also demonstrated.

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# VITAMIN A UTILIZATION STUDIES

## II. THE UTILIZATION OF VITAMIN A ALCOHOL, VITAMIN A ACETATE AND VITAMIN A NATURAL ESTERS BY THE RAT

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In the first paper of this series (Week and Sevigne, '49) it was shown that factors affecting hydrolysis play a significant part in determining the utilization of vitamin A esters by chicks. It was also shown that in general the relative utilization of differing forms of vitamin A is radically influenced by the quantity and nature of the diluent in which the vitamin is fed.

Although it was not to be expected that rats would demonstrate the effect of hydrolytic factors to the same degree as chicks, we chose to use rats for the experiments described herein because of their wide employment in the biological appraisal of vitamin A. The importance of factors which might affect the relative utilization of different forms of the vitamin was obvious, particularly when considered in relation to the U.S.P. biological assay for vitamin A.

### EXPERIMENTAL

Weanling rats were reared on the U.S.P. XIII vitamin A assay basal diet with the addition of 1.5 units of vitamin A per 10 gm of feed. The animals were housed in screen-bottom cages under uniform conditions of light, temperature, and so forth. When the rats reached 38 days of age the fat content of the diet was increased to 16% and the animals were maintained on this high fat ration — consisting of 20% casein, 52% corn-

starch, 8% brewers' yeast, 4% salt mixture, 16% hydrogenated vegetable oil, and 60 units of vitamin D and 1.5 units of vitamin A per 10 gm of ration — until they were 52 days of age. At this time they were divided into comparable groups of 10 each with respect to sex, weight and litter.

The rats were given oral supplements by means of a calibrated tuberculin syringe of either 0.1 ml or 0.4 ml of the respective vitamin A supplement daily for three successive days. Each rat received a total of 9,000 units of vitamin A for the three-day period. The rats were killed on the 4th day, or 72 hours after receiving the first supplement, and the livers were removed and stored under refrigeration until assayed for vitamin A. Control analyses indicated no loss of vitamin A during the period of storage.

The liver of each rat was assayed for vitamin A by a modification of the procedure developed by Glover et al. ('47). Vitamin A values were determined by the Carr-Price reaction using an Evelyn colorimeter standardized with the U.S.P. Vitamin A Reference Oil. These procedures are fully described elsewhere (Week and Sevigne, '49).

The vitamin A alcohol, acetate and natural ester concentrates were prepared using a male soupfin shark liver oil as the source of vitamin A. The natural ester concentrate was made by a process which does not involve the use of high vacuum distillation. The spectrophotometric and chemical characteristics of the concentrates used in this experiment fall within the limits for preparations previously described (Week and Sevigne, '49).

The dilutions of vitamin A in corn oil, jojoba seed oil and castor oil were made on the basis of vitamin A spectrophotometric units ($E\,^{1\%}_{1\,cm}$, 328 mμ × 2,000) and contained 0.5% mixed tocopherols by weight.

## DISCUSSION OF RESULTS

The analytical results are tabulated in table 1. These data were subjected to analyses of variance in order to determine the significance of the values shown.

TABLE 1

*Relationship between chemical forms of vitamin A and diluent oils, as measured by liver storage by the rat* [1]

| DILUENT | | CORN OIL | | | CASTOR OIL | | | JOJOBA SEED OIL | | | CORN OIL | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Vol. and potency of suppl. fed daily | | 0.4 ml @ 7,500 units/ml | | | 0.4 ml @ 7,500 units/ml | | | 0.4 ml @ 7,500 units/ml | | | 0.1 ml @ 30,000 units/ml | | |
| Total units fed in 3-day period | | 9,000 | | | 9,000 | | | 9,000 | | | 9,000 | | |
| Group no. | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Form of vitamin A | | Alcohol | Acetate | Ester | Alcohol | Acetate | Ester | Alcohol | Acetate | Ester | Alcohol | Acetate | Ester |
| *Sex* | *Rat no.* | | | | | | | | | | | | |
| Male | 1 | 3,350 | 3,070 | 2,910 | 2,440 | 2,560 | 2,670 | 2,500 | 2,540 | 2,090 | 3,290 | 3,520 | 3,080 |
| Male | 2 | 2,550 | 2,300 | 2,340 | 2,410 | 2,050 | 2,350 | 2,800 | 2,660 | 1,490 | 3,290 | 3,380 | 2,870 |
| Male | 3 | 2,870 | 3,200 | 2,850 | 2,450 | 3,100 | 3,260 | 2,480 | 2,890 | 1,830 | 2,650 | 3,400 | 3,350 |
| Male | 4 | 3,100 | 3,610 | 2,310 | 2,890 | 2,860 | 2,910 | 2,780 | 1,930 | 1,800 | 2,480 | 3,550 | 3,370 |
| Male | 5 | 2,890 | 3,390 | 2,940 | 2,710 | 2,490 | 2,460 | 2,580 | 2,350 | 1,930 | 3,740 | 3,600 | 3,150 |
| Male | 6 | 2,710 | 3,480 | 3,160 | 2,840 | 3,200 | 3,060 | 2,990 | 2,530 | 1,700 | 2,390 | 3,820 | 2,740 |
| Female | 7 | 3,000 | 3,150 | 2,080 | 2,720 | 2,590 | 2,500 | 2,560 | 2,900 | 2,250 | 3,010 | 4,000 | 2,820 |
| Female | 8 | 3,060 | 3,580 | 1,880 | 2,980 | 2,610 | 2,430 | 2,840 | 2,460 | 1,980 | 3,160 | 2,560 | 3,810 |
| Female | 9 | 3,380 | 4,100 | 2,780 | 3,060 | 3,280 | 2,190 | 2,460 | 3,190 | 1,430 | 2,970 | 3,910 | 2,880 |
| Female | 10 | 2,950 | 2,630 | 3,050 | 2,680 | 2,620 | 3,070 | 2,580 | 2,610 | 1,200 | 2,230 | 3,520 | 3,340 |
| Average vitamin A units per liver | | 2,986 | 3,251 | 2,630 | 2,718 | 2,736 | 2,690 | 2,657 | 2,606 | 1,770 | 2,921 | 3,526 | 3,141 |
| Average vitamin A stored (%) | | 33.2 | 36.1 | 29.2 | 30.2 | 30.4 | 29.9 | 29.5 | 28.9 | 19.7 | 32.4 | 39.2 | 34.9 |

[1] A difference of 298 units is required for significance (5% level) between any two of test groups 1 to 9, inclusive.
A difference of 379 units is required for significance (5% level) between any two of the following test groups: 1, 2, 3, 10, 11 and 12.
Control groups, receiving diluent oils only as a supplement, showed no liver storage of vitamin A.

In the case of groups fed the 0.4 ml supplement, it is possible to make 36 separate comparisons of two groups taken at a time. Seventeen of these comparisons disclose statistically significant differences, of which the following are considered to be of interest: groups 1 with 3 and 7; 2 with 3, 5 and 8; 3 with 9; 6 with 9; 7 with 9, and 8 with 9. Certain of the comparisons which do not disclose significant differences are also of interest: groups 1 with 2 and 4; 3 with 6; 4 with 5, 6 and 7; 5 with 6 and 8, and 7 with 8.

TABLE 2

*Analyses of variance of vitamin A liver storage data listed in table 1*

| SOURCE OF VARIATIONS | DEGREES OF FREEDOM | MEAN SQUARE | VARIANCE RATIO |
|---|---|---|---|
| | (1) Groups 1 to 9, inclusive | | |
| Carrier (diluent) | 2 | 2,850,468 | 25.54 |
| Individuals | 9 | 210,583 | 1.89 |
| Form of vitamin A | 2 | 2,186,234 | 19.59 |
| Carrier × form of vitamin A | 4 | 638,253 | 5.72 |
| Pooled error | 72 | 111,606 | 1.00 |
| | (2) Groups 1, 2, 3, 10, 11 and 12 | | |
| Form of vitamin A | 2 | 1,498,921 | 8.38 |
| Volume of supplement | 1 | 864,018 | 7.74 |
| Pooled error | 56 | 178,848 | 1.00 |

The comparison of group 1 with group 3 shows that factors affecting hydrolysis are of considerable importance in the utilization of vitamin A by the rat. The significantly lesser utilization of vitamin A esters versus vitamin A alcohol cannot be due to a difference of absorption per se, as the character and quantity of diluent are identical; it cannot be due to a difference of stability *in vivo,* as esters have been demonstrated to be at least equal to the free form of vitamin A in this respect; the difference must therefore be due to the effect of hydrolytic factors.

The significant difference in utilization observed in comparing group 1 with group 7 is due to the adverse effect of the

jojoba oil versus the corn oil on stability *in vivo* or absorption per se or both, in the rat. The degree to which the difference between group 3 and group 9 exceeds the difference between group 1 and group 7 demonstrates, furthermore, that the jojoba oil diluent has a significantly adverse effect, in comparison to corn oil, on the hydrolysis of vitamin A esters by the rat.

The equal utilization of the three forms of the vitamin from castor oil is interesting.

The differences in the effects of diluent variations on vitaman A acetate as compared to the effects of these variations on vitamin A natural esters are of great practical importance. These are the forms most often compared in the U.S.P. bioassay using the current U.S.P. Vitamin A Standard (vitamin A acetate). Comparisons of groups 8 with 9 and groups 5 with 6 are equally valid with respect to demonstrating the relative biological values of the two concentrates. Obviously, each comparison provides information as to relative biological efficiency, but information valid only for the specific conditions of the experiment in question.

In the cases of groups 1, 2, 3, 10, 11 and 12, it is possible to make 15 comparisons of two groups at a time. Of these, 6 disclose statistically significant differences, of which the following are considered to be important: groups 2 with 3, 3 with 12, 10 with 11, and 11 with 12.

The improved utilization of the ester forms in the smaller quantity of corn oil diluent is most interesting. The U.S.P. bioassay is conducted with a daily feeding of not more than 0.1 ml of diluent oil. The significant difference observed in vitamin A ester utilization in comparing group 3 with group 12 is due to the effect of the volume of corn oil on hydrolysis *in vivo,* as the change in volume has negligible effect on absorption per se or stability *in vivo* or both (compare group 1 with group 10).

## REMARKS

The results of these investigations show that in addition to the stability factors described by Hickman et al. ('44), and others, as affecting the relative biological response of differing

forms of vitamin A, factors influencing the hydrolysis *in vivo* of vitamin A esters may be of equal or greater significance. While the experimental results of the present study do not afford a direct quantitative measure of the factors which affect the hydrolysis of vitamin A esters under conditions of normal or sub-minimum intake of the vitamin, the data suggest certain implications regarding the experimental methods now employed in vitamin A biological assays. These methods of bioassay, namely the U.S.P. rat growth test and the Guggenheim and Koch ('44) liver storage technique, make comparisons between dissimilar chemical forms of vitamin A under one arbitrary set of experimental conditions. Our results show that certain modifications of these conditions, such as variations in the nature or quantity of the diluent used for the vitamin A supplements, radically affect the relative biological efficacy of the different chemical forms of vitamin A.

No single set of arbitrary test conditions can be shown to be more valid than another for the purpose of appraising the relative biological efficiency of differing forms of vitamin A under the multifold conditions of final use. This being true, a biological assay intended for quantitative determinations should be designed simply to measure the mol quantities of biologically active vitamin A present in test samples. If current biological assay methods were to be modified to meet this requirement, better agreement could be attained between physico-chemical and biological measurements of vitamin A, and agreement between laboratories on biological assays would be greatly improved.

As the simplest means of adapting biological assays to this desirable end, we suggest that both test sample and the Reference Standard be saponified, and that the biological test be conducted using the unsaponifiable extracts. By this method, the various esters of vitamin A are converted to a single chemical form, i.e., vitamin A alcohol; and variations in diet and diluent which may affect the stability, hydrolysis and absorption of the vitamin act in parallel fashion on both sample and Standard.

SUMMARY

1. Vitamin A liver storage measurements were used as the criterion for determining the relative utilization by the rat of the alcohol, acetate and natural ester forms of vitamin A under various experimental conditions.

2. It was shown that the relative biological efficacy of the three forms of the vitamin, although administered at equivalent unit levels, was greatly affected by the nature and quantity of the diluent present in the vitamin A supplement. Experimental conditions were established under which the relative biological response produced by the natural ester form could be varied from 59 to 105% of that produced by comparable supplements of vitamin A alcohol. It was also demonstrated that variations in the biological utilization of vitamin A acetate under diverse experimental conditions are significantly different from those of vitamin A natural esters.

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We wish to express our appreciation to Mr. A. Mittler of the California Packing Corporation and Dr. C. I. Bliss of the Connecticut Agricultural Experiment Station for their generous advice and assistance in connection with the statistical analyses; to Mr. John E. Blum of Durkee's Famous Foods for a supply of jojoba seed oil; and to Mr. H. R. Halloran and Mr. E. P. Mecchi for invaluable technical assistance.

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# PROTECTIVE EFFECTS OF SOYBEAN MEAL FOR THE IMMATURE HYPERTHYROID RAT[1]

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Available data indicate that requirements for a number of nutrients are markedly increased in the hyperthyroid animal (Drill, '43). This is particularly true for some of the B vitamins. An increased requirement for thiamine (Drill, '38; Drill and Sherwood, '38), pyridoxine and pantothenic acid (Drill and Overman, '42), folic acid (Martin, '47) and, more recently, vitamin $B_{12}$ (Nichol et al., '49; Emerson, '49; Betheil and Lardy, '49) has been demonstrated following the administration of large doses of thyroactive substances. Requirements are also increased for various unknown factors as well. These "minor vitamins" are apparently dispensable under normal conditions, or requirements for them are so small they may readily be met by amounts present in the diet or through the synthetic activity of the intestinal flora or the animal's own tissues. During hyperthyroidism, however, requirements for these substances may be increased to the extent that deficiencies occur, manifested by retarded growth or tissue pathology and preventable by the administration in appropriate amounts

[1] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 245 in the series of papers approved for publication. The views or conclusions contained in this report are those of the author. They are not to be construed as necessarily reflecting the views or indorsement of the Department of the Army.

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of the missing nutrient (Ershoff, '48a). Thus, yeast has been found to prolong significantly the average survival time of immature rats fed purified rations containing massive doses of thyroid (Ershoff and Hershberg, '45). Similar effects have been noted with desiccated and defatted whole liver (Ershoff, '47a; Betheil et al., '47). The latter material was also found to counteract completely the retardation in growth (Ershoff, '47a, '47b; Betheil et al., '47) and inhibition of ovarian development (Ershoff, '47b) of young rats fed massive doses of thyroid. Some confusion exists, however, regarding the nature of the factor or factors in liver responsible for the above effects. On rations containing soybean meal the retardation in growth caused by massive doses of desiccated thyroid or iodinated casein has been counteracted both in the rat and chick by crystalline vitamin $B_{12}$ (Nichol et al., '49; Emerson, '49). On rations containing casein as the protein and sucrose as the carbohydrate, however, the retardation in growth following administration of massive doses of thyroactive substances appears to be due, at least in part, to a deficiency of some other factor (Ershoff, '48b, '49; Betheil and Lardy, '49), which has been termed the "antithyrotoxic factor of liver." It is present in considerable concentration in the water-insoluble fraction of liver (Ershoff, '47a, '48b; Ershoff and McWilliams, '48) and is distinct from any of the known nutrients, including vitamin $B_{12}$ (Ershoff, '49). In view of the different deficiencies which are produced by thyroid feeding in animals fed the above rations, the following experiments were undertaken in an effort to determine the factor or factors in soybean meal responsible for the diverse results.

All experiments were performed with female rats of the Long-Evans strain, from 23 to 25 days of age, housed in metal cages with raised screen bottoms to minimize access to the feces. After being fed the respective experimental diets ad libitum for a period of 28 days the animals were sacrificed and the weights of the thyroids, adrenals, ovaries, kidneys and heart ventricles determined.

## EXPERIMENTAL

### *Experiment no. 1. On the antithyrotoxic properties of a full-fat soybean meal* [2]

Two basal rations were employed in this experiment, diet A and diet B (table 1). Diet A was a purified ration containing casein as the dietary protein. Diet B was similar in composition but contained a full-fat soybean meal. Each of the above diets was supplemented with U.S.P. desiccated thyroid [3] or iodinated casein [4] added in place of an equal amount of sucrose. Thyroid was incorporated in the basal rations at levels of 0.25% and 0.5%; iodinated casein at a level of 0.125%. Seventy-

TABLE 1

*Composition of experimental diets*

| DIETARY COMPONENT | DIET A | DIET B |
|---|---|---|
| | % | % |
| Casein [1] | 22.0 | 5.0 |
| Soybean meal [2] | 0.0 | 45.0 |
| Salt mixture [3] | 4.5 | 4.5 |
| Sucrose | 73.5 | 45.5 |

Vitamin supplements: to each kilogram of the above diets were added the following synthetic vitamins: thiamine hydrochloride 72 mg, riboflavin 9 mg, pyridoxine hydrochloride 15 mg, calcium pantothenate 67.2 mg, nicotinic acid 60 mg, 2-methyl-naphthoquinone 5 mg, and choline chloride 1.2 gm.[4] Each rat also received three times weekly the following supplement: cottonseed oil (Wesson) 500 mg, alpha-tocopherol acetate 1.5 mg, and a vitamin A–D concentrate [5] containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D.

[1] Vitamin test casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[2] Soya meal, El Molino Mills, Alhambra, California, a lightly roasted material containing 18 to 22% fat.

[3] Salt mixture no. 1 (Sure, '41).

[4] In view of the increased requirements for thiamine, pyridoxine and pantothenic acid in the hyperthyroid rat (Drill and Overman, '42), the B vitamins in the present experiment were administered in excessive amounts in order to assure an adequacy of these factors in the diet.

[5] Nopco fish oil concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

[2] The term "full-fat soybean meal" as employed in the present communication refers to soybean meal with an average fat content of 18 to 22%.

[3] Thyroid powder, U.S.P., Armour and Co., Chicago, Ill.

[4] Protamone, Cerophyl Laboratories, Kansas City, Missouri.

six female rats of the Long-Evans strain were selected for the present experiment. Their average weight was 46.2 gm.

Data on growth are summarized in table 2. These data indicate that the retardation in growth caused by massive doses of desiccated thyroid or iodinated casein on diet A did not occur on diet B. On the latter ration no significant difference was observed between the gain in body weight of animals fed the control ration and that of those fed a similar diet supplemented with desiccated thyroid or iodinated casein. Data on organ weights are summarized in table 3. The findings indicate that adrenal, kidney and ventricular weights, when expressed in terms of organ weight per 100 gm body weight, were increased and thyroid weight was decreased on both diets A and B in animals fed desiccated thyroid or iodinated casein. The relative increase in adrenal, kidney and ventricular weights in animals fed thyroid or iodinated casein was greater

TABLE 2

*Effects of full-fat soybean meal on the gain in body weight of immature rats fed massive doses of desiccated thyroid and iodinated casein*

The values in parentheses indicate the number of animals which survived and on which averages are based

| THYROACTIVE SUPPLEMENT | NUMBER OF RATS | | INITIAL BODY WT. | | GAIN IN BODY WT. OVER 28-DAY PERIOD [1] | |
|---|---|---|---|---|---|---|
| | Diet A | Diet B | Diet A | Diet B | Diet A | Diet B |
| | | | *gm* | *gm* | *gm* | *gm* |
| None | 8 | 8 | 46.1 | 46.0 | 100.8 ± 3.3 (8) | 102.3 ± 4.1 (8) |
| 0.25% thyroid | 10 | 10 | 45.6 | 46.0 | 60.9 ± 6.5 (9) | 107.3 ± 5.1 (10) |
| 0.5% thyroid | 10 | 10 | 46.6 | 46.1 | 45.4 ± 6.0 (8) | 104.5 ± 3.3 (10) |
| 0.125% iodinated casein | 10 | 10 | 46.4 | 47.0 | 59.4 ± 4.4 (9) | 102.8 ± 5.4 (9) |

[1] Including the standard error of the mean calculated as follows $\sqrt{\frac{\Sigma d^2}{n}} / \sqrt{n}$, where "d" is the deviation from the mean and "n" is the number of observations.

TABLE 3

*Effects of full-fat soybean meal on organ weights of immature rats fed massive doses of desiccated thyroid and iodinated casein*

| DIETARY GROUP | THYROACTIVE SUPPLEMENT | BODY WT. AFTER 28 DAYS OF FEEDING | OVARIAN WT.[1] | THYROID WT. | THYROID WT. MG/100 GM BODY WT. | ADRENAL WT. | ADRENAL WT. MG/100 GM BODY WT. | VENTRICULAR WT. | VENTRICULAR WT. MG/100 GM BODY WT. | KIDNEY WT. | KIDNEY WT. MG/100 GM BODY WT. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *gm* | *mg* | *mg* | | *mg* | | *mg* | | *gm* | |
| A | None | 146.9 | 42.8 ± 2.5 | 9.5 | 6.5 | 35.8 | 24.4 | 564 | 384 | 1.575 | 1.072 |
| A | 0.25% thyroid | 107.8 | 25.3 ± 2.7 | 5.9 | 5.5 | 36.3 | 33.7 | 809 | 750 | 2.041 | 1.893 |
| A | 0.5% thyroid | 92.7 | 22.7 ± 2.9 | 5.5 | 5.9 | 29.0 | 31.3 | 739 | 797 | 2.137 | 2.305 |
| A | 0.125% iodinated casein | 105.4 | 20.0 ± 1.5 | 6.1 | 5.8 | 31.2 | 29.6 | 811 | 769 | 2.039 | 1.935 |
| B | None | 148.3 | 43.1 ± 3.2 | 9.9 | 6.7 | 37.7 | 25.4 | 543 | 366 | 1.573 | 1.061 |
| B | 0.25% thyroid | 153.3 | 40.7 ± 6.0 | 8.8 | 5.7 | 43.9 | 28.6 | 729 | 476 | 2.188 | 1.427 |
| B | 0.5% thyroid | 150.6 | 28.8 ± 2.6 | 8.3 | 5.5 | 42.3 | 28.1 | 767 | 509 | 2.234 | 1.480 |
| B | 0.125% iodinated casein | 150.8 | 38.8 ± 6.2 | 8.7 | 5.8 | 43.9 | 29.1 | 750 | 497 | 2.143 | 1.421 |

[1] Including the standard error of the mean. See footnote 1, table 2.

on diet A than on diet B. Ovaries appeared immature both in weight and microscopic appearance in animals fed diet A supplemented with desiccated thyroid or iodinated casein. On diet B, however, 7 out of 10 rats fed thyroid at a level of 0.25% and 6 out of 9 animals fed iodinated casein had apparently normal ovaries. When thyroid was fed at a level of 0.5%, only two out of 10 rats had ovaries which appeared normal. No abnormalities in weight or microscopic appearance were observed in the ovaries of rats fed similar rations with thyroid or iodinated casein omitted. The protective effect of full-fat soybean meal was also evident in respect to gross appearance. On diet A animals fed thyroid or iodinated casein had an unthrifty appearance, with ruffled fur and occasionally alopecia; on diet B, however, animals fed thyroid or iodinated casein were indistinguishable grossly from the normal controls.

Basal metabolic rates were determined on the 25th day of the experiment for animals fed the various soybean rations.[5] No attempt was made to obtain readings for animals fed diet A supplemented with thyroid or iodinated casein in view of the high susceptibility of such animals to heart failure following excitation. Previous findings from this laboratory indicate that rats maintained on synthetic rations supplemented with massive doses of thyroid readily succumb following unaccustomed handling. In preliminary tests, 7 out of 8 rats fed diet A supplemented with 0.5% thyroid died in the respiration chamber during the course of oxygen determinations; on the other hand, animals fed similar rations supplemented with 10% whole liver or 10% yeast survived similar handling (Ershoff, '47a).

Eight rats in each experimental group were tested. The apparatus used was of a closed circuit type, with a capacity of 2 l (Mason, Kryder and Winzler, '49). Carbon dioxide was absorbed with sodium hydroxide, and oxygen consumption was determined from pressure changes recorded by means of a

[5] We are indebted to Mr. George Kryder of the Department of Biochemistry and Nutrition, University of Southern California, for the determinations of basal metabolic rates.

water manometer. The respiration chambers were kept at 27°C. and readings obtained were corrected to standard temperature and pressure. Food was removed from the animals' cages the evening prior to the metabolism test. At least 6 successive 5-minute intervals were recorded for each animal, with care being taken to measure oxygen consumption when animal activity was at a minimum. The two lowest readings were employed in calculating the basal rate. Results are summarized in table 4. They indicate that full-fat soybean meal did not counteract the rise in basal metabolic rate caused by

TABLE 4

*Effects of desiccated thyroid and iodinated casein on the basal metabolism of immature rats fed diets containing full-fat soybean meal*

| DIETARY GROUP | THYROACTIVE SUPPLEMENT | NUMBER OF ANIMALS | $O_2$ CONSUMPTION ML./HR./100 GM BODY WT.[1,2] |
|---|---|---|---|
| A | None | 8 | 139.0 ± 2.4 |
| B | None | 8 | 133.7 ± 3.4 |
| B | 0.25% thyroid | 8 | 227.4 ± 4.3 |
| B | 0.5% thyroid | 8 | 246.2 ± 6.4 |
| B | 0.125% iodinated casein | 7[3] | 229.9 ± 8.2 |

[1] Including the standard error of the mean. See footnote 1, table 2.

[2] This method of reporting these data is used, rather than "0.75 power of the body weight" or some similar expression, inasmuch as there was substantially little difference in the body weights of the animals.

[3] Originally 8 rats were present in this group; one animal died during the determination.

desiccated thyroid or iodinated casein. It is apparent, therefore, that the protective effect of soybean meal as observed in the present experiments was an antithyrotoxic effect, and was not due to neutralization, destruction or impaired absorption of the thyroid hormone.

The presence of a goitrogenic agent in soybeans has been demonstrated by McCarrison ('33) and confirmed by Sharpless ('38) and others (Sharpless et al., '39). Thyroid enlargement was demonstrated by these workers only on low iodine diets, since the goitrogenic effects of soybean were counteracted by iodine (Sharpless et al., '39). In the present experi-

ment no goitrogenic effects such as increase in thyroid weight or reduction in basal metabolic rate resulted from the feeding of full-fat soybean meal, a finding presumably due to the high iodine content of the diet employed.

### *Experiment no. 2. Comparative effects of raw and autoclaved full-fat soybean meal on the growth of immature thyroid-fed rats*

The following experiment was undertaken to determine the effects of autoclaving on the growth-promoting properties of

TABLE 5

*Comparative effects of raw and autoclaved full-fat soybean meal on the gains in body weight of immature rats fed massive doses of desiccated thyroid*

The values in parentheses indicate the number of animals which survived and on which averages are based

| GROUP | NUMBER OF ANIMALS | INITIAL BODY WT. | GAIN IN BODY WT. AFTER 28 DAYS OF FEEDING [1] |
|---|---|---|---|
| | | *gm* | *gm* |
| I | 6 | 44.6 | 99.8 ± 7.1 (6) |
| II | 8 | 44.7 | 96.8 ± 4.4 (7) |
| III | 8 | 44.5 | 93.3 ± 4.4 (7) |

[1] Including the standard error of the mean. See footnote 1, table 2.

full-fat soybean meal for the immature thyroid-fed rat. Three experimental groups were employed. Group I was fed diet B (table 1); group II received diet B plus 0.5% desiccated thyroid [6] (with thyroid added in place of an equal amount of sucrose); group III was fed a diet similar to that fed group II but containing soybean meal which had been autoclaved for 30 minutes at a pressure of 15 pounds. Twenty-two female rats with an average weight of 44.6 gm were selected for the present experiment. The results are summarized in table 5. No significant differences in gain in body weight were observed for

[6] See footnote 3, page 261.

animals fed any of the diets employed. It is apparent, therefore, that autoclaving under the conditions of the present experiment did not impair the growth-promoting effect of full-fat soybean meal for the immature thyroid-fed rat.

### *Experiment no. 3. Comparative effects of low-fat soybean flour and full-fat soybean meal on the growth of immature thyroid-fed rats*

This experiment was undertaken to determine the comparative effects of low fat soybean flour and full-fat soybean meal on the gains in body weight of immature rats fed massive doses of desiccated thyroid. Three soybean sources were employed: (1) a full-fat soybean meal;[7] (2) an extracted soybean flour containing 4% fat;[8] and (3) an expeller-processed soybean flour containing 7% fat.[9] The rations fed were similar to diet B (table 1) but differed in the source of the soybean product employed. Diets were fed with and without desiccated thyroid,[10] which was administered at a level of 0.5% in place of an equal amount of sucrose. Forty-eight female rats with an average weight of 46.0 gm were selected for this experiment. The results are summarized in table 6.

The findings indicate that a direct correlation exists between the fat content of the soybean component of the diet and its antithyrotoxic effect. The gain in body weight of immature rats fed massive doses of desiccated thyroid was greatest on a diet containing full-fat soybean meal (18 to 22% fat) and least on a ration containing extracted soybean flour (4% fat). Values for the diet containing "expeller" soybean flour (7% fat) lay between these two. When thyroid was omitted from the above rations, no significant difference in growth was observed on any of the diets employed. It would appear, therefore, that the antithyrotoxic properties of full-fat soybean meal were

[7] Soya meal, El Molino Mills, Alhambra, Calif. This material was similar to that employed in experiments 1 and 2 but came from another lot.

[8] Extracted soy flour (4% blended), A. E. Staley Mfg. Co., Decatur, Ill.

[9] Soy Flour Lo-Fat, A. E. Staley Mfg. Co., Decatur, Ill.

[10] See footnote 3, page 261.

correlated with its fat content, and that soybean oil or some factor in it was responsible for the antithyrotoxic effect.

### *Experiment no. 4. Comparative effects of vitamin $B_{12}$, extracted liver residue and soybean oil on the gain in body weight of immature rats fed massive doses of desiccated thyroid*

Previous findings indicate that vitamin $B_{12}$ counteracts the growth-retarding effect of massive doses of thyroid when fed

TABLE 6

*Comparative effects of low fat soybean flour and full-fat soybean meal on gain in body weight of immature rats fed massive doses of desiccated thyroid*

The values in parentheses indicate the number of animals which survived and on which averages are based

| SOYBEAN COMPONENT IN DIET | THYROID | NUMBER OF ANIMALS | INITIAL BODY WT. | GAIN IN BODY WT. AFTER 28 DAYS OF FEEDING [1] |
|---|---|---|---|---|
| | % | | gm. | gm. |
| Extracted soybean flour 4% fat | 0.5 | 10 | 45.9 | 56.5 ± 4.6 (8) |
| "Expeller" soybean flour 7% fat | 0.5 | 10 | 46.0 | 70.4 ± 3.6 (10) |
| Full-fat soybean meal 18–22% fat | 0.5 | 10 | 46.1 | 97.1 ± 4.8 (8) |
| Extracted soybean flour 4% fat | 0.0 | 6 | 45.8 | 88.0 ± 5.1 (6) |
| "Expeller" soybean flour 7% fat | 0.0 | 6 | 46.0 | 87.4 ± 3.6 (6) |
| Full-fat soybean meal 18–22% fat | 0.0 | 6 | 46.0 | 99.2 ± 3.7 (6) |

[1] Including the standard error of the mean. See footnote 1, table 2.

in conjunction with a diet containing soybean meal (Emerson, '49). The present experiment was undertaken to determine the comparative effects of vitamin $B_{12}$ and extracted liver on the gain in body weight of immature rats fed massive doses of thyroid in conjunction with diets containing either casein or low-fat soybean flour as the principal source of protein. In view of the experiments reported above, in which full-fat soy-

bean meal exerted an antithyrotoxic effect, animals on each of the above rations were also fed soybean oil (at a level corresponding to its content in diets containing full-fat soybean meal) in an effort to determine what effect this material might have on the gain in body weight of immature thyroid-fed rats.

Two basal rations were employed, diet A and diet C. Diet A was a purified ration containing casein as the dietary protein; diet C was similar in composition but contained a low fat soybean flour.[11] These diets were identical with diets A and B (table 1), except that a low fat soybean flour replaced the full-fat soybean meal of diet B. Five experimental groups were fed each of the basal rations. Group I was fed the basal ration alone; group II the basal ration plus 0.5% desiccated thyroid;[12] group III the basal ration plus 0.5% desiccated thyroid plus 30 μg vitamin $B_{12}$ per kilogram of diet;[13] group IV the basal ration plus 0.5% desiccated thyroid plus 10% extracted liver residue;[14] and group V the basal ration plus 0.5% desiccated thyroid plus 10% cold pressed soybean oil. The dietary supplements and the desiccated thyroid were added in place of an equal amount of sucrose.

Eighty female rats with an average weight of 44.8 gm were selected for this experiment. The effects of the various diets on body and ovarian weights are summarized in table 7.

The findings indicate that animals fed diet A differed significantly from those fed diet C following the administration of massive doses of thyroid. On diet A the retardation in growth of thyroid-fed rats (group II) was completely counteracted by extracted liver residue (group IV), partially counteracted by soybean oil (group V), and unaffected by vitamin $B_{12}$ (group III). On diet C the growth retardation of thyroid-fed rats (group II) was completely counteracted by all three sup-

[11] See footnote 9, page 267.

[12] See footnote 3, page 261.

[13] The vitamin $B_{12}$ employed in the present experiment was obtained from Merck and Co., Rahway, N. J.

[14] Extracted liver residue, Wilson Laboratories, Chicago, Ill. This product consists of the coagulated, water-insoluble material remaining after the removal of the extractable water-soluble substances.

TABLE 7

*Comparative effects of vitamin $B_{12}$, extracted liver residue and soybean oil on body and ovarian weights of immature rats fed massive doses of desiccated thyroid*

The values in parentheses indicate the number of animals which survived and on which averages are based

| DIETARY SUPPLEMENT | THYROID | NUMBER OF RATS | | INITIAL BODY WT. | | GAIN IN BODY WEIGHT OVER 28-DAY PERIOD [1] | | OVARIAN WT.[1] | |
|---|---|---|---|---|---|---|---|---|---|
| | | Diet A | Diet C | Diet A | Diet C | Diet A | Diet C | Diet A | Diet C |
| | % | | | *gm* | *gm* | *gm* | *gm* | *mg* | *mg* |
| None | 0.5 | 8 | 8 | 44.9 | 45.0 | 48.4 ± 3.9 (5) | 68.3 ± 3.0 (6) | 24.0 ± 2.2 | 25.9 ± 2.3 |
| Vitamin $B_{12}$ | 0.5 | 8 | 8 | 44.7 | 45.0 | 51.8 ± 3.6 (6) | 93.4 ± 4.1 (8) | 25.1 ± 2.8 | 35.6 ± 4.1 |
| Extracted liver residue | 0.5 | 8 | 8 | 44.4 | 44.8 | 102.6 ± 4.0 (6) | 99.0 ± 5.1 (7) | 48.8 ± 6.1 | 28.7 ± 3.2 |
| Soybean oil | 0.5 | 8 | 8 | 45.0 | 45.0 | 78.4 ± 4.3 (7) | 92.6 ± 3.7 (7) | 27.8 ± 2.6 | 29.5 ± 3.3 |
| None | 0.0 | 8 | 8 | 44.4 | 44.6 | 99.2 ± 4.7 (8) | 91.2 ± 4.6 (8) | 43.1 ± 2.7 | 39.8 ± 3.9 |

[1] Including the standard error of the mean. See footnote 1, table 2.

plements (groups III, IV and V). Furthermore, thyroid-fed rats on diet C gained significantly more weight than similar animals (group II) on diet A. In the absence of dietary thyroid (group I), no significant differences in growth were observed between rats fed either of the basal diets.

Differences in ovarian weights were noted between rats fed diets A and C. On diet A ovaries appeared immature both in weight and microscopic appearance in groups II, III and V. Among the thyroid-fed rats, only those fed extracted liver residue (group IV) had ovaries which were similar in weight and in histological appearance to those of the normal controls (group I). On diet C, however, ovarian weight was retarded in all thyroid-fed rats (groups II, III, IV and V). Kidney, adrenal and ventricular weights were increased on both diets A and C in all thyroid-fed rats.

### *Experiment no. 5. Comparative effects of soybean oil and other vegetable and animal fats on the gains in body weight of immature rats fed massive doses of desiccated thyroid*

The basal ration employed in this experiment was diet A (table 1). Animals were fed diet A plus 0.5% desiccated thyroid [15] plus 10% of each of the following fats: olive oil, peanut oil, cottonseed oil, wheat germ oil, corn oil, soybean oil, hydrogenated cottonseed oil [16] and lard.[17] Control groups were fed diet A alone and diet A plus 0.5% desiccated thyroid. Thyroid and the various fats were incorporated in the above diets in place of an equal amount of sucrose.

[15] See footnote 3, page 261.

[16] Crisco.

[17] The fats were obtained from the following sources: olive oil, Strohmeyer and Arpe Co., New York, N. Y.; peanut oil, Planters Edible Oil Co., San Francisco, Calif.; cottonseed oil, Wesson Oil and Snowdrift Sales Co., New Orleans, La.; wheat germ oil, VioBin Corporation, Monticello, Ill.; corn oil, Corn Products Refining Co., Argo, Ill.; soybean oil, House of Better Living, Los Angeles, Calif.; Crisco, Procter and Gamble, Cincinnati, Ohio; and lard, Rath Packing Co., Waterloo, Iowa.

Eighty-seven female rats with an average weight of 45.9 gm were selected for this experiment. Diets were prepared biweekly and stored under refrigeration.

The effects of the various diets on gain in body weight are summarized in table 8. These indicate that massive doses of

TABLE 8

*Comparative effects of vegetable and animal fats on the gains in body weight of immature rats fed massive doses of desiccated thyroid*

The values in parentheses indicate the number of animals that survived and on which averages are based

| SUPPLEMENT ADDED TO BASAL RATION | THYROID | NUMBER OF ANIMALS | INITIAL BODY WT. | GAIN IN BODY WT. OVER 28-DAY PERIOD [1] |
|---|---|---|---|---|
| | % | | gm | gm |
| None | 0.5 | 15 | 46.0 | 44.7 ± 2.9 (9) |
| Olive oil | 0.5 | 8 | 46.1 | 67.7 ± 1.6 (6) |
| Peanut oil | 0.5 | 8 | 45.6 | 69.6 ± 1.4 (6) |
| Cottonseed oil | 0.5 | 8 | 45.9 | 70.1 ± 4.1 (7) |
| Wheat germ oil | 0.5 | 8 | 45.8 | 73.8 ± 3.0 (5) |
| Corn oil | 0.5 | 8 | 45.8 | 74.6 ± 3.9 (7) |
| Soybean oil | 0.5 | 8 | 46.1 | 74.7 ± 3.5 (7) |
| Crisco | 0.5 | 8 | 45.9 | 76.2 ± 4.5 (6) |
| Lard | 0.5 | 8 | 46.0 | 82.9 ± 3.9 (5) |
| None | 0.0 | 8 | 45.4 | 101.8 ± 5.7 (8) |

[1] Including the standard error of the mean. See footnote 1, table 2.

thyroid caused a marked retardation in the gains in body weight of immature rats fed a low fat diet. The growth-retarding effect of thyroid was partially counteracted by the increased administration of fat. All fats tested increased growth significantly over that observed in thyroid-fed rats on the low

fat diet, but gain in body weight was most marked for the animals fed lard. In view of the small number of rats employed, it is doubtful if the differences in growth among animals fed the various fat-containing diets were statistically significant. Although increasing the fat content of the diet augmented the gains in body weight of immature rats fed massive doses of thyroid, it did not counteract other effects of thyroid administration, such as inhibition of ovarian development or increase in kidney, adrenal and ventricular weights. Soybean oil was not superior to other fats in its ability to promote growth in the immature thyroid-fed rat.

## DISCUSSION

It is becoming increasingly apparent that the effects of thyroid feeding are dependent to a considerable degree on the composition of the basal ration. It is well known that massive doses of thyroid cause a marked retardation in growth in the immature rat. Recent findings indicate, however, that the growth-retarding effect of thyroid may be completely counteracted by the administration of defatted and desiccated whole liver (Ershoff, '47a, '47b; Betheil et al., '47) and other animal tissues such as defatted and desiccated heart, kidney, placenta, duodenum, thymus and brain (Ershoff, '48b).

Massive doses of thyroid have also been found to inhibit ovarian development in the immature rat (Ershoff, '45). This effect may also be counteracted by the administration of defatted and desiccated whole liver (Ershoff, '47b). Liver feeding, however, does not prevent the rise in oxygen consumption nor the increase in adrenal, kidney and ventricular weights following thyroid feeding, nor will it prevent the attendant reduction in ventricular creatine concentration (Ershoff, '47a). It is apparent that the protective effects of liver noted above are *antithyrotoxic* effects, and not due to neutralization, elimination or destruction of the thyroid hormone.

In the present series of experiments data were obtained indicating that full-fat soybean meal also exerts an antithyrotoxic

effect in the immature female rat. The protective effects of this substance are due in part to its fat content, but there appears to be at least one factor in soybeans other than their fat content which also plays a part in counteracting symptoms of thyrotoxicity in the immature rat.

A number of reports have appeared concerning the beneficial effects of dietary fat for the hyperthyroid animal. Abelin and co-workers (Abelin, '26; Abelin and Kürsteiner, '28; Abelin, Knuchel and Spichtin, '30) observed that increasing the fat content of the diet counteracted in part the rise in basal metabolic rate and the reduction in liver glycogen following thyroid feeding. Berg ('34) found that large amounts of vegetable fats lowered the metabolic rates of hyperthyroid dogs. Similar findings have been observed with respect to certain animal and vegetable fats and the hyperthyroid rat (Munoz, '44; Spalloni Cialdea, '45; Hoffmann, Hoffmann and Talesnik, '45). Available data indicate that the protective effects of fat noted above were due, at least in part, to certain unsaturated fatty acids.

Keeser ('38) reported that sodium oleate and linoleic acid protected against the rise in oxygen consumption following administration of the thyroid hormone; while Guerra ('47) observed that the protective effects of animal and vegetable fats against the rise in basal metabolic rate following thyroid feeding were correlated with the degree of unsaturation of the various fats. Similar data concerning the protective effects of certain unsaturated fatty acids on the hyperthyroid animal have been reported by Zain ('36, '37). This investigator found that linoleic acid, but not stearic acid, would prevent reduction in liver glycogen following administration of massive doses of thyroid to the female rat. Values obtained with oleic acid lay between these two. Linoleic acid also protected against loss in weight following thyroid feeding. Although some of the effects noted above may have been due, at least in part, to an increased caloric intake or to the ingestion of fat-soluble vitamins or other nutrients present in the various fats, the data nevertheless indicate that fat per se, and certain fatty

acids in particular, may modify the effects of excessive amounts of thyroid hormone.

The results of the present experiments provide further data concerning the beneficial effects of fat on the hyperthyroid animal. It was observed that increasing the fat content of the diet partially counteracted the growth retardation of immature rats fed massive doses of thyroid in conjunction with a low fat diet. A number of fats were tested at a level of 10% in the diet for their growth-promoting effects on the immature hyperthyroid rat. Olive oil, peanut oil, cottonseed oil, wheat germ oil, corn oil, soybean oil, hydrogenated cottonseed oil and lard were all found to cause a gain in body weight significantly greater than that observed in thyroid-fed rats on a control low fat ration. They did not, however, counteract other effects of thyroid administration, such as inhibition of ovarian development or increase in kidney, adrenal and ventricular weights.

No data are available concerning the mechanism by which fat exerts its growth-promoting effect on the immature hyperthyroid rat. It is possible that requirements for certain fatty acids are increased in the hyperthyroid animal to the extent that a deficiency of these nutrients is precipitated on diets low in fat. The gain in body weight following the administration of various fats might be regarded under these circumstances as due to the correction of a nutritional deficiency. The butyl alcohol residue of extracted liver residue, however, which contains virtually no fat, is far more effective than any of the above fats in counteracting the growth retardation of hyperthyroid rats on a low fat diet (Ershoff, '48b). It would appear, therefore, that the growth-promoting effect of fat on the hyperthyroid rat is due to some factor other than the correction of a fatty acid deficiency. It is possible that fat may stimulate the synthesis of an antithyrotoxic factor by the intestinal flora. Whipple and Church's finding ('35) that lard exerts a favorable effect on the intestinal synthesis of thiamine in the rat may be pertinent in this regard. No data are available indicating to what extent differences in growth

on the various rations may have been due to differences in caloric intake.

The present experiments indicate that full-fat soybean meal completely counteracts the growth retardation of immature rats fed massive doses of desiccated thyroid. Gain in body weight was considerably reduced, however, when low fat soybean flour was fed in place of the full-fat soybean meal. When thyroid was omitted from the above rations, no significant differences in growth were observed on any of the diets employed. The retardation in growth of thyroid-fed rats on a diet containing low fat soybean flour was completely counteracted by the administration of 10% soybean oil, added in place of an equal amount of sucrose. Similar results were obtained, however, with the administration of 10% extracted liver residue or crystalline vitamin $B_{12}$ when fed at a level of 30 μg per kilogram of diet.

The significance of these findings is not readily apparent. Although the protective effects of extracted liver residue might conceivably be due to a bound form of vitamin $B_{12}$, it is difficult to account for the equal effectiveness of soybean oil. If the assumption is made that the growth retardation of hyperthyroid rats on a diet containing low fat soybean flour is due to a vitamin $B_{12}$ deficiency, an interpretation in accord with previous findings (Emerson, '49), it follows that soybean oil either: (1) decreases body requirements for vitamin $B_{12}$; (2) stimulates the synthesis of vitamin $B_{12}$ by the intestinal flora or the animal's own tissues; or (3) contains significant amounts of a substance with vitamin $B_{12}$ activity. No data are available for determining which of these alternatives, if any, is correct.

In agreement with earlier findings (Ershoff, '48b, '49), vitamin $B_{12}$ in the present experiments failed to counteract the growth retardation of hyperthyroid rats fed a diet low in fat and containing casein as the dietary protein. Animals fed extracted liver residue under similar experimental conditions, however, gained as much weight as the normal controls. These results indicate that the growth-promoting effect of

extracted liver residue is not due to its vitamin $B_{12}$ content. The gains in body weight of thyroid-fed rats receiving soybean oil was intermediate between those of animals fed vitamin $B_{12}$ and those of rats fed extracted liver residue.

Data on ovarian weight also indicate that the protective effects of extracted liver residue, under the conditions of the above experiment, were due to some factor other than vitamin $B_{12}$. The ovaries of thyroid-fed rats on the vitamin $B_{12}$ supplement were immature both in weight and microscopic appearance. Similar findings were obtained with animals fed soybean oil. The ovaries of rats fed extracted liver residue, however, were indistinguishable grossly and in histological appearance from those of the normal controls. The protective effects of extracted liver residue were presumably due to an unidentified nutrient, which has been termed the "antithyrotoxic factor of liver." Available data indicate that this factor is distinct from any of the known nutrients, including vitamin $B_{12}$ (Ershoff, '47a, '48b, '49; Betheil et al., '47).

It is apparent from the above findings that massive doses of thyroid can precipitate a deficiency of either vitamin $B_{12}$ or the "antithyrotoxic factor of liver" in the immature female rat, depending on the composition of the basal ration. When immature female rats were fed a low fat diet containing casein as the dietary protein, the retardation in growth following thyroid feeding could be counteracted by extracted liver residue but not by vitamin $B_{12}$, indicating that it was due to a deficiency of the "antithyrotoxic factor of liver." When immature female rats were fed a similar diet containing low fat soybean flour as the dietary protein, the resulting growth retardation was due to a deficiency of vitamin $B_{12}$. The question immediately arises of why animals fed the low fat soybean flour did not develop a deficiency of the "antithyrotoxic factor of liver" and, conversely, why rats fed the casein-containing ration did not develop a deficiency of vitamin $B_{12}$.

It was observed in the course of the present experiments that when immature female rats were fed massive doses of thyroid in conjunction with a low fat diet, they gained signifi-

cantly more weight on a ration containing low fat soybean flour as the dietary protein than they did on a diet containing purified casein. Since the amount of thyroid in both diets was identical, it is apparent that the soybean flour either: (1) contained one or more factors which counteracted in part the growth-retarding effects of thyroid; or (2) stimulated the synthesis of such a factor or factors by the intestinal flora or the animal's own tissues.

Although part of the growth-promoting effect of low fat soybean flour may have been due to its fat content (7%), it would appear that it contains at least one additional factor which is qualitatively similar to, if not identical with, the "antithyrotoxic factor of liver." The failure of thyroid-fed rats on a soybean-containing ration to develop a deficiency of the "antithyrotoxic factor of liver" may be due to the presence of such a factor. Recent findings (Rubin and Bird, '47) indicate that diets high in soybean meal increase body requirements for vitamin $B_{12}$. The combination of such a diet and an increased vitamin $B_{12}$ requirement resulting from thyroid feeding (Betheil and Lardy, '49) would appear to be primarily responsible for the appearance of a vitamin $B_{12}$ deficiency in hyperthyroid rats on a soybean-containing ration.

The pretest dietary regime is also of importance in conditioning responses to thyroid feeding. Since significant amounts of vitamin $B_{12}$ can be stored in the tissues of the young rat during the period of pregnancy and lactation (Emerson et al., '49), the occurrence of a vitamin $B_{12}$ deficiency and its extent following thyroid feeding is dependent in part on the vitamin $B_{12}$ content of the pretest dietary regime. The growth retardation of hyperthyroid rats fed a diet low in fat and containing casein as the dietary protein was completely counteracted in the present experiments by extracted liver residue. Vitamin $B_{12}$ under similar experimental conditions showed no growth-promoting activity. It would appear, therefore, that the growth retardation noted above was due entirely to a factor or factors other than vitamin $B_{12}$. Apparently, sufficient amounts of vitamin $B_{12}$ were stored by the animals from their

pretest dietary regime to meet any increase in vitamin $B_{12}$ requirements resulting from thyroid feeding. Betheil and Lardy ('49) recently reported that vitamin $B_{12}$ *partially* counteracted the growth retardation of male rats fed thyroid-containing rations similar to those described above. It would appear that these workers were dealing with a deficiency of both vitamin $B_{12}$ and the "antithyrotoxic factor of liver." Further work is needed to determine to what extent differences in sex, strain and pretest dietary regime were responsible for the diverse results obtained by Betheil and Lardy and by the present author.

## SUMMARY

Full-fat soybean meal completely counteracted the growth retardation of immature rats fed massive doses of desiccated thyroid or iodinated casein. It did not counteract, however, the attendant reduction in basal metabolic rate.

The antithyrotoxic effects of full-fat soybean meal were correlated in part with its fat content. Growth was retarded in hyperthyroid rats fed similar diets but containing low fat soybean flour in place of the high fat soybean meal.

The retardation in growth of immature hyperthyroid rats on a diet containing low fat soybean flour was completely counteracted by the administration of either soybean oil, extracted liver residue or crystalline vitamin $B_{12}$. When immature rats were fed a similar diet but containing casein as the dietary protein, the growth retardation following thyroid feeding was completely counteracted only by extracted liver residue. Soybean oil showed some counteracting activity; vitamin $B_{12}$ was inactive.

Increasing the fat content of the diet augmented significantly the gains in body weight of hyperthyroid rats fed a diet low in fat and containing casein as the dietary protein. Both animal and vegetable fats were active in this respect.

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## MEAD JOHNSON AND COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1950 Award of $1000.00 established by Mead Johnson and Company to promote researches dealing with the B-complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute in the spring of 1950.

The Award will be given to the laboratory or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year, January 1 to December 31, the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Committee of Judges, nominations for this award for work published in 1949 must be in the hands of the Chairman of the Nominating Committee by January 15, 1950. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

W. H. Sebrell, Jr.
*Experimental Biology and Medicine Institute*
*National Institutes of Health*
*Bethesda, Maryland*

Chairman, Nominating Committee

## BORDEN AWARD IN NUTRITION

Nominations are solicited for the 1950 Award of $1000.00 and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the judges may recommend that it be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute in the spring of 1950. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 15, 1950. The nominations should be accompnaied by such data relative to the nominee and his research as will facilitate consideration for the award.

L. A. MAYNARD
*Cornell University, Ithaca, New York*

CHAIRMAN, NOMINATING COMMITTEE

## OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of $1000.00 established by the Nutrition Foundation, Inc. for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojurning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. Nominations for the 1950 Award, accompanied by data relative to the accomplishments of the nominee, must be sent to the Chairman of the Nominating Committee before January 15, 1950.

H. E. Carter
*University of Illinois*
*Urbana, Illinois*

Chairman, Nominating Committee